TITLE : THE EFFECT OF DIPHENYLHYDANTOIN UPON THE STEM CELLS OF THE MURINE TERATOCARCINOMA CELL LINE PC 13

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CHAPTER 1.

TERATOGENIC AGENTS : ANTICONVULSANT DRUGS

1.1 TERATOGENIC AGENTS

A teratogenic agent is a drug, chemical, virus, physical agent or a deficiency state that, by acting during the embryonic or fetal period, alters morphology or subsequent function in the post natal period (Shepard 1979). Generally, a drug is considered a teratogenic agent if it causes, when administered to a pregnant patient in the critical period of organogenesis, congenital malformations which are specific and unexpected from the general rules of genetics. Such malformations would need to be proved to be independent of the disease of the mother who required the drug, and consistent in nature and incidence. The malformation would need to be consistent with the data from animal experiments demonstrating the relationship between the drug and the malformation (Nakan 1980).

Analysis of the above type of information has led to the tabulation of a number of agents said to be ' known ' teratogens in man (Table 1.).

TABLE 1. KNOWN TERATOGENIC AGENTS IN MAN (Shepard 1979).
(causing specified % of congenital malformations)

RADIATION (1%)

therapeutic

radioiodine

atomic weapon

INFECTIONS (2-3%)

rubella

cytomegalovirus

herpes simplex (oral and genital ?)

toxoplasmosis

syphilis

varicella (?)

Venezuelan equine encephalitis

MATERNAL METABOLIC IMBALANCE (1-2%)

endemic cretinism

diabetes (?)

phenylketonuria

virilizing tumours

alcoholism

DRUGS AND ENVIRONMENTAL CHEMICALS (4-6%)

androgenic hormones

aminopterin and methylaminopterin

cyclophosphamide

busulfan

thalidomide

mercury

chlorobiphenyl

diethylstilbestrol

diphenylhydantoin and trimethadione

coumadin derivatives

Other defined causes of congenital abnormalities include genetic transmission (20%) and chromosome aberrations (3 - 5%) ; however, the cause of the majority (65 - 70%) of developmental defects is yet unknown (Wilson 1977) although probably produced by a combination of several gene defects and environmental agents. This phenomenom called multifactorial inheritance suggests that there is an underlying genetic predisposition to form congenital malformations, produced by the small effects of many genes interacting with an environmental trigger, such as a drug. This genetic predisposition has two major components, a predisposition to react adversely to a given agent and a predisposition to one or more forms of maldevelopment (Nora 1981). Each of these may have far reaching implications when one considers adequate screening tests and the evaluation of teratogenic agents.

Drugs and environmental chemicals demand special attention since if they are found to be the causes of developmental defects they can be avoided during pregnancy and so reduce the incidence (approximately 3%) of congenital anomaly in newborns (Shepard 1979).

1.2 THE PREGNANT WOMAN AND DRUG EXPOSURE

Most women are exposed to environmental agents, medications, drugs and occupational hazards on a daily basis (Doering and Stewart 1978). Women continue to take large quantities of drugs during pregnancy. A Scottish study in 1973 (Forfar et al) reported that 82% of women were prescribed drugs other than iron

during pregnancy and that these women took an average of four different preparations. In the same study, self-prescribed medication was taken in addition by 65% of the women. In Sweden, Boethius (1978) had shown that drug consumption increased during pregnancy when compared with the pre-pregnant or non-pregnant states. Because of the normal high incidence of multiple drug exposure in pregnant women, retrospective evaluation of a drug for teratogenic toxicity is difficult.

In the case of epileptic patients, many are on multiple drug therapy and it is difficult to determine which drug or combination of drugs may be responsible for the defects observed (Sullivan and McElhatton 1976).

1.3 EPILEPSY AND ANTICONVULSANT DRUGS : DIPHENYLHYDANTOIN

Anticonvulsants may be amongst the drugs administered to a pregnant epileptic woman. Epilepsy is one of the most prevalent neurological disorders. The essence of epilepsy is focal abnormal paroxysmal discharge of cells of the central nervous system. There are clinical (behavioural) and electrical seizures, and recurring episodes make up the disorder called epilepsy. Brain damage and heredity appear to play aetiological roles, however much is still unknown about its cause. The essential brain mechanisms, neurotransmitters and related phenomena are still unknown. The incidence of epilepsy is approximately 0.5% in all populations surveyed (Dreifuss 1980).

Many patients with epilepsy receive diphenylhydantoin (DPH) which was first introduced in 1938 (Merrit and Putnam).

Despite the introduction of some twenty other anticonvulsant drugs, DPH is still the preferred drug of many for the treatment of grand mal epilepsy (Kutt and Penry 1974).

In the brain, DPH has little effect on the electrical activity of an epileptic focus, instead, it acts to contain the spread of propagated electrical activity by inhibiting post-tetanic potentiation, a method of facilitated spread of neural activity which occurs transiently following a sustained, high-frequency burst of nerve cell activity, as during an epileptic seizure (Conomy 1978).

1.4 ANTICONVULSANTS AND A REPORTED TERATOGENIC EFFECT

The association between epilepsy, anticonvulsant drugs and birth defects was first proposed by Janz and Fuchs in 1964. Data from isolated reports of affected humans are now supported by research on both humans and animals. It was only recently that clinical reports have suggested that a specific pattern of abnormalities exists in the offspring of mothers receiving hydantoin anticonvulsants during pregnancy (Hanson and Smith 1975). The link between a specific drug and a characteristic defect remains unsettled (Paulson and Paulson 1981).

The first suspicion of DPH teratogenesis was raised in 1963 (Muller - Kuppers) and five years later Meadow (1968) noted that among mothers of cleft lip and/or cleft palate babies, a higher than expected number had taken anticonvulsants during pregnancy. In the wake of these observations, many

investigations were undertaken and resulted in a number of case series and population studies which have suggested that DPH may induce up to a three-fold increase in major anomalies in the children of women who take DPH regularly during the first trimester (Annegers et al 1978; Frederick 1973; Knight and Rhind 1975; Monson et al 1973 and Hanson et al 1976).

1.5 ARE ANTICONVULSANT DRUGS INDEED TERATOGENIC ?

Although there is considerable literature on the effect DPH may exert on the developing embryo and fetus, the teratogenicity of anticonvulsants remains controversial (Finnel and Chernoff 1982) There are several ways to interpret the increased frequency of congenital anomalies in the babies of treated epileptic mothers (Lakos and Czeizel 1977), and these have been summarized as follows :-

Table 2. POSSIBLE CAUSES OF THE INCREASED FREQUENCY OF CONGENITAL ANOMALIES IN THE BABIES OF TREATED EPILEPTIC MOTHERS

Conclusion	Details and Author(s) of Report
a. due to the effect of the epileptic condition	The offspring of women suffering from a seizure disorder are at a slightly increased risk for congenital malformations regardless of any prenatal exposure to anticonvulsants. There was no appreciable difference in the incidence of infants with defects born to treated as opposed to

Continued/...

Table 2. Continued/...

Conclusion	Details and Author(s) of Report
see a.	non-treated epileptic mothers. (Shapiro et al 1976; Frederick 1973; Meyer 1973, and Starreveld-Zimmermann 1973).
b. due to the anticonvulsant drug(s) taken during pregnancy	Researching the same literature as Shapiro et al (1976), these authors searched for a particular ' pattern of malformations ' referred to as the Fetal Hydantoin Syndrome, which they concluded as being the consequence of embryonic and fetal exposure to the hydantoins. In support of this conclusion, most of the known teratogens in man give rise to such patterns of multiple defect. (Hanson et al 1976).
	The malformation rate in babies born to epileptic mothers in a pre-anticonvulsant therapy period (1929 - 1945) was compared with a later era (1946 - 1968) during which anti-convulsants were available. A marked increase in congenital malformations since the introduction of anticonvulsant drugs was found. (Visser, Huisjes and Elshove 1976).

Table 2. Continued/...

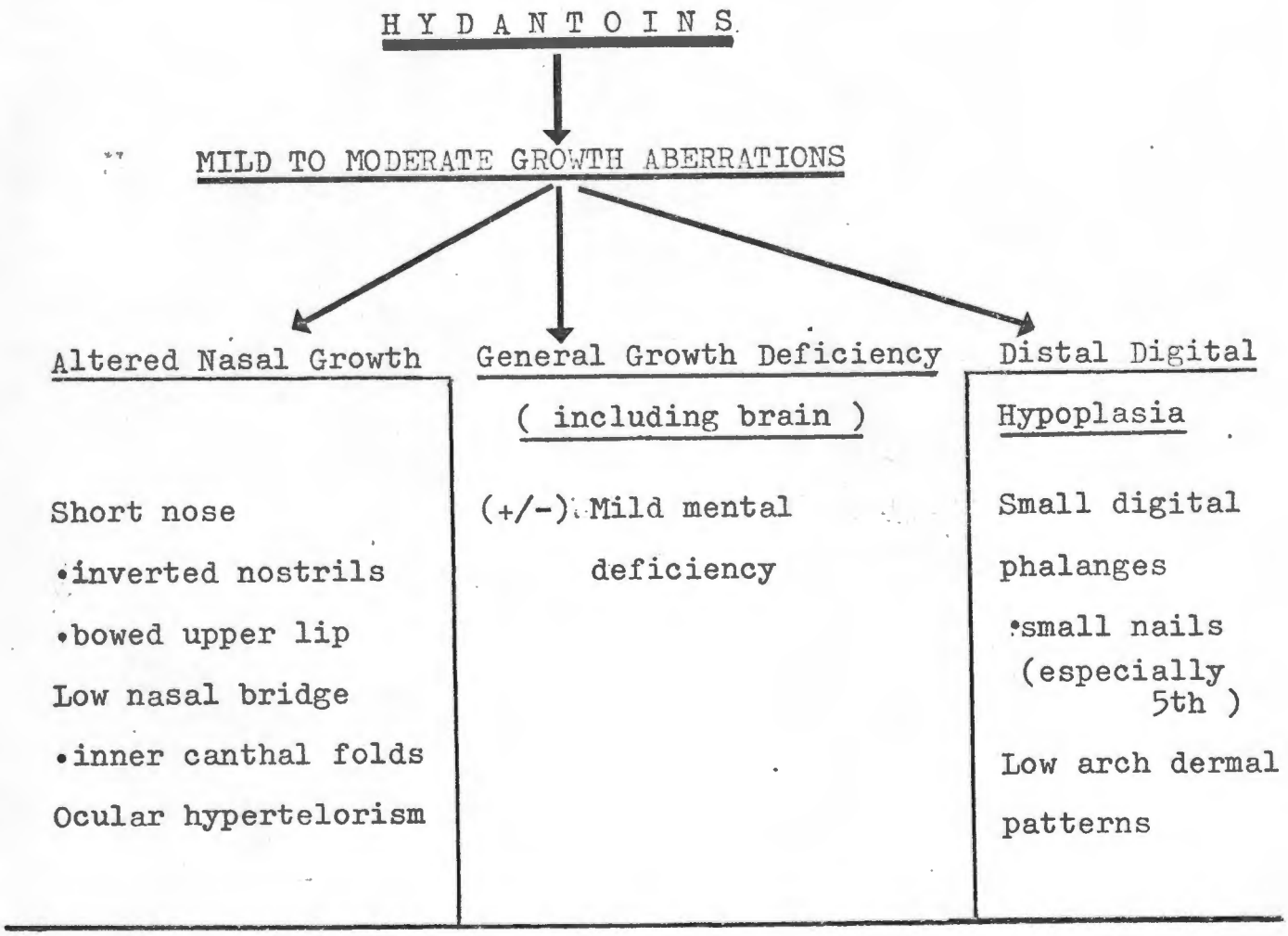
Conclusion	Details and Author(s) of Report
see b.	In opposition to the interpretation noted in part a. other authors have stated that when examining the frequency of congenital disorders in the infants born to treated and non-treated epileptics, there is a significantly increased frequency in the former group. (Lakos and Czeizel 1977).
c. problems associated with epilepsy e.g. Socio-economic status	Epilepsy may result in social and financial difficulties which could lead to a lower than average standard of living and detrimental effects on the general health of a pregnant woman, making infections more frequent, which might ultimately lead to a teratogenic effect. (Lakos and Czeizel 1977).

Livingsone and colleagues (1979) reported that they were not convinced that the evidence relative to the teratogenicity of anticonvulsive agents was conclusive. The data suggest that both epilepsy and DPH may be risk factors for the development of the congenital malformations (Wilson 1978).

1.6 THE FETAL HYDANTOIN SYNDROME IN MAN

There has been a great deal of variability in the effects of DPH on the fetus in terms of both severity and number of defects, however, Figure 1. sets forth the most consistent effects, which constitute the predominant features of the Fetal Hydantoin Syndrome (FHS), a " broad multisystem of abnormalities " first described by Hanson and Smith in 1975.

Figure 1. THE MOST CONSISTENT FEATURES IN INDIVIDUALS WITH THE FETAL HYDANTOIN SYNDROME (Smith 1979)



In terms of human handicap, deficits such as mental retardation are often far more devastating than a structural malformation which could possibly be corrected surgically, such as cleft palate. Studies by Hanson et al (1976) have shown that there is a 10% risk of a mild to moderate mental retardation in the children exposed and a 30% incidence of one or more of the malformations described in Figure 1. Most organ tissues or body systems can be affected, hence babies exposed to DPH in utero merit a close overall scrutiny for defects of morphogenesis.

DPH has also been stated as causing alterations of post - natal development and behaviour, acting as a ' behavioural teratogen ' (Barlow 1982). The appearance of behavioural disorders may be months or even years removed from the precipitating event.

Animal studies designed to determine the teratogenic potential of DPH have demonstrated that the type and degree of malformations observed are dependent on such variables as the time of exposure and dose (Paulson and Paulson 1981).

1. 61 EXPOSURE TIME

Following the exposure of pregnant mice to DPH for a short time during organogenesis, single malformations such as cleft lip, cleft palate, urogenital anomalies, ocular and neural defects were observed in the offspring (Finnel and Chernoff 1982). In humans, by virtue of the nature of the malformations that occur, such as cleft lip and cardiac defects, it is clear that the hydantoins may cause serious problems in morphogenesis

during the first trimester of pregnancy (Dudley 1980). During this stage of cell differentiation and organogenesis, the developing tissues are more sensitive to adverse influences on structural development than mature tissues and the developing organism is at its greatest susceptibility. Different organs commence and complete their development at different times and the susceptibility to a teratogen applied at a specific time will differ from organ to organ depending on the stage of development concerned, resulting in selective vulnerability (Paulson and Paulson 1981).

Whether there is a serious risk during middle or late gestation remains to be resolved (Dudley 1980). These latter stages of prenatal development are the period of maturation and growth. All organs have been formed structurally and interference with late development will tend to lead to deformations, growth retardation or functional disturbances rather than malformations. The biochemical, physiological and behaviour changes result in functional abnormalities which may not be detectable at birth (Goldman 1979). In experimental animals it has been found that during the latter part of the fetal period the conceptus becomes sensitive to transplacental carcinogens (Shepard 1979). Where DPH is given towards the end of pregnancy, the clotting factors in the infant may be depressed sufficiently to cause bleeding, leading to clotting disoders in the newborn infant (Dukes 1980).

The type of defect produced cannot be precisely predicted on the basis of the time of insult (Shepard 1979). Taking into account the critical period for human congenital disorders

caused by anticonvulsants, the period from the fourth to the tenth week of gestation may be considered dangerous (Lakos and Czeizel 1977).

1.62 DOSE

It is generally accepted that any drug given in large enough amounts will adversely affect fetal development. This action usually occurs through deleterious effects on maternal health and is expressed as either embryo/fetus death, or fetal growth and development retardation (Shepard 1979). Many of the warnings on drug package inserts about potential teratogenicity are related to this phenomenom. When extrapolating the findings from animal experiments to humans, it is important to take into consideration the ratio (on a per kilogram basis) between the teratogenic dose in the animal and the therapeutic dose in the human.

It has been recommended (Wilson 1973) that for in vivo animal experiments, the dosage of the test substance should be maximal but not interfere with the health of the pregnant animal, in-order that a fetotoxic response be obtained. In most animal research reports, the dosages of all medications have been ten-fold or more than the comparable normal human dose, but malformations also occur at near therapeutic levels (20μg/ml) (Paulson and Paulson 1981).

It has been suggested (Organisation Scientific Group 1967) that one negative control group and three dosage levels should be used, the highest of which should cause maternal toxicity.

The lowest dosage should be well below the toxic dosage and should ideally cause effects similar to those caused in man by the therapeutic dosages of the medicine. The intermediate dose should ideally lie logarithmically between the highest and lowest dosages. These guidelines were accepted and adapted for the in vitro experiments performed in this investigation.

1.7 SUMMARY

DPH has been found to be teratogenic in mice and is believed to be so in humans, with multifactorial inheritance determining the occurrence and severity of the resultant congenital defects. Data suggest that it is both the epileptic condition and DPH which may be the risk factors for the development of the pattern of abnormalities known as the Fetal Hydantoin Syndrome, although it is now generally accepted that anticonvulsants can by themselves damage the fetus.

CHAPTER 2.

DIPHENYLHYDANTOIN TERATOGENICITY AND PHARMACOKINETIC FACTORS

2.1 TERATOLOGICAL INVESTIGATION AND PARAMETERS INVOLVED

Analysis of the pharmacological or toxicological effect of a drug, such as DPH, thought to have a teratogenic action, would always consist of an investigation of two types of parameters (Neubert et al 1978). Pharmacokinetic factors involve all parameters associated with the agent being ' handled ' by the organism. This includes the rates of absorption, distribution, metabolic conversion and elimination. These factors are the only ones which can modify the degree of an effect to be evaluated. In addition, there are pharmacodynamic factors which will be considered in the following chapter but briefly, they specify the kind of pharmacological or toxic action at the target with the resulting effects typical for the agent applied, and also the elucidation of its mode of action (Neubert et al 1978).

2.2 ABSORPTION AND DISTRIBUTION OF DPH

DPH is an organic acid of low solubility which is usually given orally, the daily dose totalling up to 6 mg/kg . The drug is not rapidly absorbed from the gastrointestinal tract and ordinarily is slowly metabolized. Its mean half - life in plasma is twenty two hours after an oral dose. These characteristics permit once - daily drug administration in many patients. Effective serum levels of DPH are 10 - 20 μg/ ml (Conomy 1978), however blood levels are markedly lower during pregnancy than before pregnancy or after delivery. This may be

due to alterations in absorption, excretion and metabolism of the drug, the dilution effects of increasing weight and extracellular fluid volume, or fetal metabolism of a part of the anticonvulsant dose (Nakan 1980).

Once absorbed, 90% of the drug is reversibly bound to plasma proteins and α_2- globulins, and the DPH is distributed to the extracellular fluid of all organs, with the brain, muscle, adipose tissue, liver, pituitary and adrenal glands having high affinity for the drug (Conomy 1978).

2.3 DRUGS AND CONDITIONS WHICH AFFECT THE PHARMACOKINETICS AND TOXICITY OF DPH

In addition to DPH, a pregnant woman may be exposed to drugs other than DPH, or have a condition which causes an increase in plasma levels of DPH thus leading to prolongation of DPH half-life or DPH intoxication (Table 3.). With regard to the dose - teratological effect relationship, an increase in the probability of the offspring having a birth defect could result. Selective and reduced drug administration and/or treatment of the condition (other than epilepsy), might lead to DPH levels remaining at a constantly low but therapeutically effective level. Conversely, drugs other than DPH, or a condition (other than epilepsy) might lead to a shortened DPH half-life or to under-dosage, resulting in the ineffective treatment of the epileptic state (Table 3.). Should this lead to an increase in epileptic seizures, this in itself might have a teratogenic effect, whilst the concentration of the putative teratogenic agent (DPH) is reduced (Shapiro et al 1976).

Table 3. DRUGS AND CONDITIONS WHICH AFFECT THE PHARMACOKINETICS AND TOXICITY OF DPH (Conomy 1978)

Drugs which cause increased plasma DPH levels, prolongation of DPH half - life or acute DPH intoxication

Bishydroxycoumarin (dicumarol)
Chloramphenicol (Chloromycetin)
Chlorpromazine (Thorazine)
Chlordiazepoxide (Librium)
Diazepam (Valium)
Disulfiram (Antabuse)
Estrogens
Ethosuximide
Halothane (Fluothane)
Isoniazid
Methylphenidate (Ritalin)
Phenylbutazone (Butazolidin)
Phenyramidol
Prochlorperazine (Compazine)
Propoxyphene (Darvon , SK-65)
Sulfaphenazole (Orisul)
Sulthiame (Trolone)
Valproic acid

Conditions which cause increased plasma DPH levels, prolongation of DPH half - life or acute DPH intoxication

Hepatic insufficiency — i.e., hepatitis, cirrhosis, genetic (hepatic) parahydroxylation defect

Drugs which cause low plasma DPH levels, shorten DPH half - life or lead to underdosage

Phenobarbitone (rarely clinically significant)
Carbamazepine
Ethanol
Antacids

Conditions which cause low plasma DPH levels, shorten DPH half - life or lead to underdosage

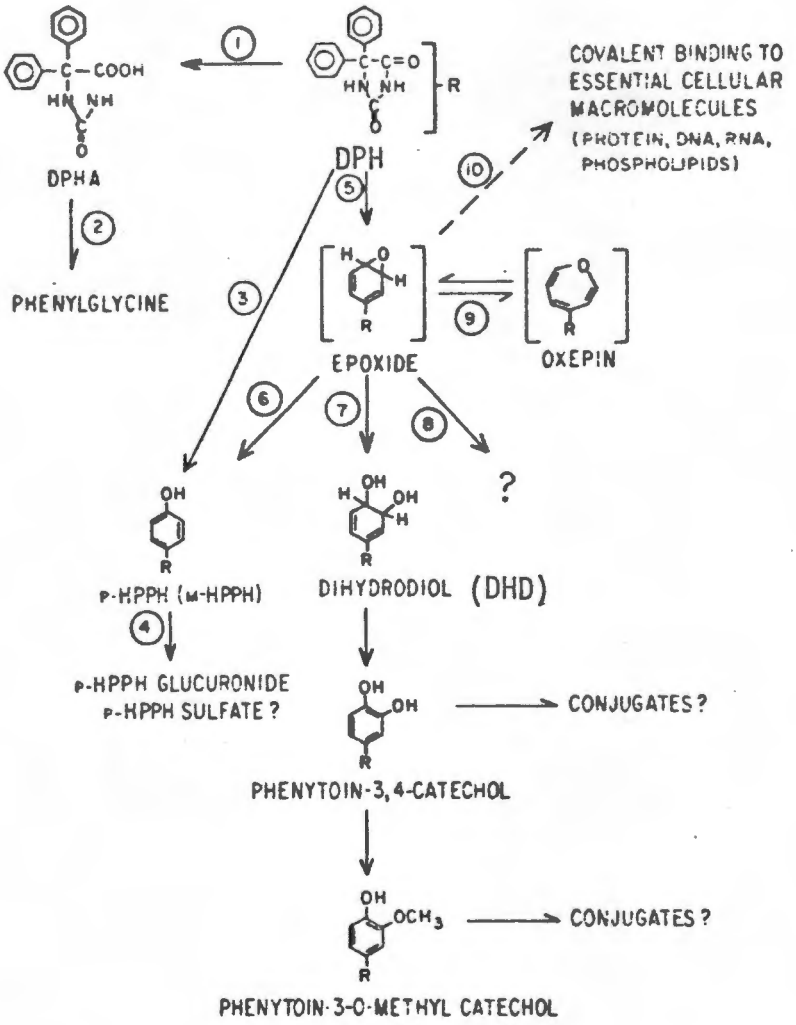
Chronic renal disease
Newborn state
Intestinal absorptive defects
Intramuscular DPH administration

2.4 DPH METABOLITES : FORMATION AND EXCRETION

Metabolism of DPH is primarily by liver enzyme parahydroxylation. In man, the metabolism of DPH is extensive, only a minor portion being excreted unchanged in the urine (Butler 1957; Maynert 1960).

Figure 2. PROPOSED BIOTRANSFORMATION OF DPH IN VIVO

(Wells and Harbison 1980)



Abbreviations:

DPH = diphenylhydantoin
DPHA = diphenylglycine-α- aminodiphenyl acetic acid
m-HPPH = 5-(3'-hydroxyphenyl)-5-phenylhydantoin
p-HPPH = 5-(3'-hydroxyphenyl)-5-phenylhydantoin
DHD = 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl) -5-phenylhydantoin

Figure 2. Continued/...

Key :

STEP	COMMENT
①	The hydantoin ring is opened yielding DPHA.
②	DPHA is deamidated to phenylglycine.
③	DPH is hydroxylated, via mixed function oxidase enzymes, directly to p-HPPH and m-HPPH.
④	The hydroxylated metabolites are excreted primarily as glucuronide conjugates and possibly sulphate conjugates.
⑤	DPH metabolism involves bioactivation to a toxic arene oxide (epoxide) intermediate by hepatic microsomal cytochrome P-450 - catalysed monooxygenase enzymes.
⑥	Subsequent deactivation of the arene oxide probably occurs by a spontaneous nonenzymatic isomerization to the hydroxylated metabolites. Indirect evidence for an intermediate arene oxide includes detection of the DHD metabolite in the urine of mice, rats, monkeys, humans (Harbison et al 1977), and human neonates (Horning et al 1971). In general, arene oxides are the presumptive intermediates in the oxidation of most aromatic hydrocarbons, as reflected by the occurrence of the isomerization step to phenol metabolites, the formation of glutathione (GSH) conjugates and the formation of DHD, catechols and their conjugates (Jerina and Daly 1974).
⑦	This involves the formation of DHD catalysed by hepatic microsomal epoxide hydrase enzymes.
⑧	Additional metabolic pathways of minor quantitative but substantial toxicologic importance are also possible.
⑨	The arene oxide may also exist in equilibrium with its oxepin tautomer, their concentrations depending on their relative stabilities (Jerina et al 1970b). This may be true for DPH, although neither species has been identified (Harbison 1978).
⑩	Finally, if there is either an overwhelming increase in arene oxide production (Step 5), or a decrease in arene oxide detoxification (Steps 6,7, and 8), the highly reactive arene oxide will be free to bind to essential cellular macromolecules causing alterations in cell function or cell death.

Table 4. A QUALITATIVE AND QUANTITATIVE SUMMARY OF DPH METABOLIC PRODUCTS FOUND IN URINE SAMPLES

Species	Metabolite		Reference
Man	p - HPPH	The major metabolite, appears in urine largely as a conjugate with glucuronic acid and normally accounts for 50 - 75% of the daily DPH dose.	Dudley 1980
Rat Mouse Cat		Found in urine in greater amounts than m - HPPH.	Chang and Glazko 1972
Man	m - HPPH	Normally represents 5 - 13% of the total urinary HPPH composition.	Dudley 1980
Dog		The major metabolite.	Chang and Glazko 1972
Man	DHD	3 - 12% of DPH daily dose.	Horning et et al 1971
Man		Up to 20% of DPH daily dose. Genetic variations in the conjugation reaction of DHD and/or variations in enzyme activity of the DHD-Catechol pathway, may affect amounts.	Dudley 1980
Mouse		Detected in urine.	Harbison et al 1977
Monkey Rat		The major metabolite.	Chang and Glazko 1972
Rat		First discovered in the rat.	Chang et al 1970

DPHA if present in human urine probably does not exceed 0.2 - 0.3% DPH daily dose (Butler et al 1976). A unique N- glucuronide metabolite of DPH has also been reported in man, and other minor metabolites include phenytoin-3,4-catechol and phenytoin-3-O-methyl catechol, but no information is available on the quantitative aspects of these urinary products in man (Dudley 1980).

2.5 PRODUCTION OF THE ARENE OXIDE INTERMEDIATE AND DHD BY DPH METABOLISM

The mechanisms of the reactions leading to the formation of the monophenolic metabolites (p-HPPH and m-HPPH) are of particular interest because these biotransformations could involve a highly reactive arene oxide intermediate (Figure 2. Steps 3 and 6). The arene oxide is also thought to be an intermediate in the formation of DHD, therefore it is presumed that a patient produces a considerable amount of this ' obligatory ' and possibly teratogenic intermediate in the metabolism of DPH (Jerina and Daly 1974).

It has been postulated that free DHD is a ' masked ' or devious form of the arene oxide intermediate (Dudley 1980). If associations could be made between unusually high free DHD levels and DPH toxicity, this concept could provide an adequate explanation of how a highly reactive arene oxide could be produced principally at one site within the body and exert its adverse effect at some distant site. The arene oxide is presumably an extremely short-lived and reactive compound that has eluded attempts at detection by gas chromatography - mass spectrometry (Horning et al 1976).

2.6 THE DISTRIBUTION OF DPH AND ITS METABOLITES IN FETAL TISSUES

Parent drug and metabolites have been identified in different amounts in placenta, and in various fetal tissues (Egger 1978). The total concentration of DPH and metabolites in fetal tissues varied from 10 - 20% of the levels of the unconjugated fraction in human plasma, whereas levels in the placenta were found to be slightly higher than those in maternal plasma. Metabolites were also identified in human fetal brain, liver and muscle tissues. The polar metabolites of DPH were also demonstrated to be present in the fetal tissues examined and were thus able to pass the placental, as well as the fetal blood- brain barrier.

The arene oxides produced in maternal liver may be stable enough to cross the placenta into the fetus, or may tautomerize to a more stable oxepin which crosses the placenta and isomerizes back to the reactive arene oxide. Some arene oxide could also be produced directly by fetal tissue metabolism of DPH, although this route would not be likely to contribute significantly to overall arene oxide production (Wells and Harbison 1980).

2.7 SUMMARY

The pregnant state, drugs other than DPH, and medical conditions may affect the pharmacokinetics and toxicity of DPH, and these must be considered when deciding DPH dosage for the effective treatment of the epileptic condition and the prevention of teratogenicity.

CHAPTER 3.

DIPHENYLHYDANTOIN TERATOGENICITY AND PHARMACODYNAMIC FACTORS

3.1 MECHANISM OF DPH TERATOGENICITY

It is still not certain whether or not DPH produces its teratogenic effect by acting through the parent compound or a toxic metabolite (as a reactive intermediate or as a final metabolite). There now follows a tabulated summary of the relevant research work on this subject.

Table 5. TERATOGENICITY OF DPH : PARENT COMPOUND

SPECIES (Author)	EXPERIMENTAL CONDITIONS	CONCLUSION
Mouse (Harbison and Becker 1974)	Phenobarbitone stimulated DPH metabolism, so producing an increase in the quantity of DPH metabolites in a short period of time and was demonstrated to antagonize DPH-induced resorptions and anomalies. In contrast, SKF 525-A pretreatment which inhibits DPH metabolism, produced a greater plasma concentration of DPH (maternal and fetal) and slowed maternal excretion of DPH to cause a potentiation of DPH-induced resorptions and anomalies.	The results indicated that under conditions that apparently stimulated DPH metabolism, DPH-embryotoxicity was decreased; when conditions favoured inhibition of the metabolism of DPH (parent compound) it was enhanced.

Continued/...

Table 5. Continued/...

SPECIES (Author)	EXPERIMENTAL CONDITIONS	CONCLUSION
Mouse (Harbison and Becker 1974)	The authors investigated the relative teratogenic potency of the hydroxylated and degradative metabolites of DPH when compared with that of DPH per se. They studied hydroxyphenyl phenyl hydantoin, ∝- amino-diphenyl acetic acid, diphenylhydantoic acid and DPH effects on the fetuses.	The metabolites did not affect fetal growth and were generally less fetocidal than DPH. These results suggest that DPH was the active teratogen.
Rhesus Monkey (Gabler and Hubbard 1972)	A study was made of the distribution and metabolism of DPH (parent compound) and its metabolites in maternal and fetal tissues.	It was concluded that if the distribution pattern of DPH and its metabolites in cardiac tissues was indicative of the distribution pattern for most tissues (other than kidney), it would appear that DPH may be the prime candidate to play the role of a teratogen as the parent compound. However, more direct evidence is warranted.

Numerous carcinogens and mutagens have been shown to require metabolic activation to ultimate metabolites inorder to exert toxic actions (Jerina and Daly 1974). It is reasonable to infer therefore that teratogenic effects of some chemicals might also result from enzymatic activation. The toxic effects of DPH may be due to a metabolite or intermediate in metabolism, rather than due to the parent compound (Blake and Martz 1980).

Table 6. TERATOGENICITY OF A PRODUCT OF DPH METABOLISM

SPECIES (Author)	EXPERIMENTAL CONDITIONS	CONCLUSION
Mouse (Blake and Martz 1980)	Administration of ^{14}C - DPH to pregnant mice and measurement of the non-extractable radioactivity presumed to be covalently bound to macroproteins, was determined in whole embryo and placenta. The amount of bound label increased when pregnant mice were pretreated with an inhibitor of epoxide hydratase, the enzyme responsible for degradation of the arene oxide. There also resulted an increase in the teratogenic activity of the drug.	These findings support the hypothesis that DPH owes its teratogenic effect to the production of a reactive arene oxide metabolite which binds to macromolecules critical for normal development. Confirmation of this postulate in humans could only be obtained by examining tissue metabolite patterns which poses ethical and logistical problems.

Continued/...

Table 6. Continued/...

SPECIES (Author)	EXPERIMENTAL CONDITIONS	CONCLUSION
Mouse (Harbison and Becker 1974)	In a study of the comparative embryotoxicity of DPH and some of its metabolites in mice, it was found that when examining the number of anomalies and resorptions after treatment of the mother with DPH, the diphenylhydantoic acid, diphenylglycine and p - HPPH metabolites do not account for DPH's teratogenicity.	The parent drug or a metabolite or intermediate other than those examined is responsible for DPH teratogenicity.
Mouse (Blake and Martz 1980)	Reviewing the work by Harbison and Becker (1974), the authors attributed the significant decrease in fetal body weight and crown-rump and transumbilical measurement in the offspring of DPH-treated mothers to the teratogenic action of the arene oxide intermediate, and not to the parent compound.	The arene oxide intermediate is responsible for DPH teratogenicity.
Mouse (Martz et al 1977)	In an examination of DPH teratogenesis, there appeared a correlation between the fetotoxic effect and the covalent binding of a putative arene oxide metabolite in gestational tissue.	Arene oxide is a teratogenic agent in the mouse.

The major metabolites of human DPH metabolism, viz. p- HPPH, m- HPPH, and DHD also occur in other animals including the mouse. An important determinant of DPH levels and its rate of metabolism is the genetic variation in the enzyme activity of individuals, which may account for the variations in fetotoxic response.

Evidence suggests that the arene oxide intermediate in DPH metabolism is probably the toxic agent in DPH teratogenicity and by a covalent binding to the essential cellular macromolecules, affects cellular development. DHD, rather than being just a detoxification product of DPH metabolism may be a ' masked ' form of the arene oxide which travels via DHD to exert effects at a distant site.

Metabolites of DPH have been found to pass the placenta and even the fetal blood brain barrier indicating that these barriers are not able to prevent the fetus from being exposed to these products. The arene oxide may cross directly or indirectly via an Oxepin tautomer. All fetal tissues are exposed and their development may be interfered with leading to congenital anomalies.

3.2 MECHANISMS OF TERATOGENICITY AT THE MOLECULAR AND CELLULAR LEVELS

A firm binding between the causing agent and the special cellular target molecules may directly cause embryotoxic effects by one or more of the following mechanisms or 'initial events' (Wilson 1979b) :-

1. Chromosome breaks, nondisjunction etc.,
2. Mutation (gene)
3. Mitotic interference
4. Altered nucleic acid integrity or function
5. Lack of normal precursors, substrates, etc.,
6. Altered energy sources
7. Changed membrane characteristics
8. Osmolar imbalance
9. Enzyme inhibition

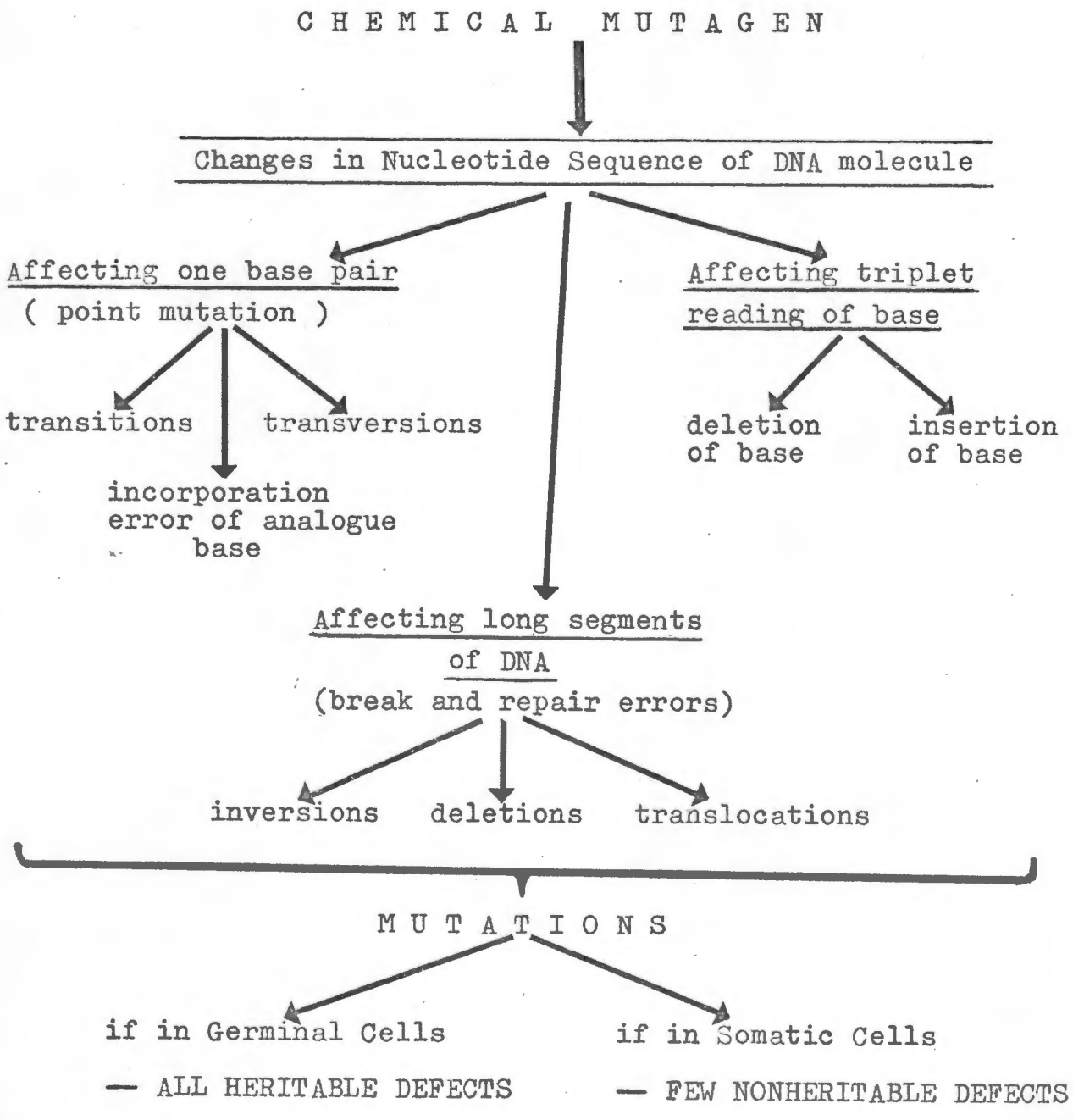
These changes in a developing system are often not readily apparent because they are at subcellular or molecular levels. To become better manifest the postulated mechanisms must lead to grosser 'secondary 'events which are more readily demonstrable cellular and tissue alterations that constitute recognizable events of abnormal embryogenesis and are as follows (Wilson 1979b):-

1. Excessive or reduced cell death
2. Failed cell interactions
3. Reduced biosynthesis
4. Impeded morphogenetic movement
5. Mechanical disruption of tissues

3.3 MUTATION AS THE 'INITIAL EVENT'

The various ways in which nucleotide sequences in the DNA molecule may be changed in the production of mutations is illustrated in Figure 3.

Figure 3. CHEMICAL MUTAGENESIS (Wilson 1973).



3.31 THE RELATIONSHIP BETWEEN A MUTATIONAL (INITIAL) EVENT, A CYTOTOXIC (SECONDARY) EVENT AND THE MANIFESTATION OF CONGENITAL ANOMALIES

DNA rearrangements occur in normal development which code for the differentiation of specific cell communication proteins. These proteins are responsible for the proper functioning of growth control in a multicellular organism. DNA - damaging agents (mutagens) could possibly induce DNA repair enzymes, some of which may catalyse illegitimate genome rearrangements, thus leading to a change in cell growth, proliferation and differentiation, giving rise to congenital anomalies (Wintersberger 1982).

It has been postulated (Newlon 1975) that some CNS malformations are related to the human equivalent of the mouse T locus using the indirect evidence that the T locus is linked to the mouse histocompatibility locus H - 2, which is clearly analogous to the HLA locus in man. If some cases of congenital malformations were determined by gene(s), within a system akin to the T locus, the abnormalities would be linked to the HLA locus. Therefore, some human congenital anomalies may correspond to those controlled by mutation at the T locus in the mouse. The restricted region of the complex T locus in the mouse appears to contain an important centre controlling the steps of early embryogenesis. The T locus in the mouse is located on chromosome 17, near the complex H - 2 major histocompatability locus to which it may be functionally related, in that both may be concerned with the recognition of events. Mutants at the T locus of the mouse form a series of recessive lethals, each of which act at a specific stage

of development (Newlon 1975).

The observed effects of t alleles on embryogenesis suggest that the role of these genes is to specify cell surface components required for cellular interactions at precise stages. The T locus is thought to be related to gene(s) which code for the F 9 antigen which is present on the membrane of early mouse embryos, spermatozoa and whole male germinal cells but not on adult somatic tissue. F 9 antigen in human cells is found in a distribution very similar to that previously described in the mouse. It supports the existence of a human equivalent to the T locus. However, the genetic control of F 9 antigen in man, especially its linkage relationship with the HLA locus remains to be demonstrated (Fellous 1979).

It could therefore be considered a possibility that DPH may exert its teratogenic effect by causing the ' initial event ' of a mutation at the human equivalent of the T locus where possibly the F 9 antigen is genetically controlled, and so cause altered membrane constitution and affect the cellular interactions which are so vital for normal tissue development. The ' secondary event ' of altered cell proliferation rates, reduced cell size, altered cell morphology, or effects on cell migration and cell differentiation may result. The possibility of a mutagenic effect from DPH cannot be ruled out (Bartoshesky and Pashayan 1982).

3.32 MUTAGENICITY OF AN INTERMEDIATE/PRODUCT OF DPH METABOLISM

Arene oxides have been shown to be mutagenic to mammalian cell cultures, Drosophila, bacteriophages and strains of Salmonella typhimurium (Jerina and Daly 1974).

The discussion will now centre on the proposal that arene oxides are the bioactivated agents responsible for the teratogenicity of DPH in causing mutagenic and carcinogenic effects (Jerina and Daly 1974). However, the possibility that the other metabolites of DPH, or the parent compound are active in producing these effects cannot be excluded.

The arene oxides are prime suspects for the bioactivated intermediates responsible for DPH teratogenicity (Wells and Harbison 1980). In addition to isomerizing to phenols, they react readily with a variety of nucleophiles, including such cellular macromolecules as DNA, RNA and proteins which are critical for normal development. Reacting as such, arene oxides metabolically derived from drugs can be putative teratogenic agents and toxic effects can be correlated with the extent of binding (Jerina and Daly 1974).

Either the devious form of the arene oxide intermediate, or a mischievous substance that is bioactivated to a toxic DHD - arene agent may react with cellular constituents to interrupt normal cellular processes (Dudley 1980).

Deactivation of the arene oxide appears to be the major factor in reducing DPH teratogenicity. The enzymes responsible for the formation and destruction of the arene oxide are likely to be under separate control and be differentially responsive to environmental factors, therefore it is possible to postulate that individual human fetuses would have varying sensitivity to the teratogenic action of DPH, depending on the genotype and maternal exposure to other drugs, environmental pollutants and so forth. It is possible that some affected babies have a combination of genetic and environmental factors which lead to enhanced levels of the arene oxide intermediate (Wells and Harbison 1980).

3.33 MUTATION AND CARCINOGENESIS

A variety of chemicals have been implicated as causative agents in carcinogenesis. Each of the chemical carcinogens that have been thoroughly studied have been found to bind covalently with DNA, RNA and protein of the target tissue (Miller,1970). The covalent binding of these bioactivated intermediates (arene oxides) to intracellular macromolecules provides a molecular basis for carcinogenicity (Jerina and Daly 1974). For most cancers some of the steps involved in their initiation must surely be mutational because the correlation between mutagenicity and carcinogenicity is so strong (Cairns 1979).

DPH has been associated with malignant lymphoma (Hyman and Sommers 1966), and hepatic necrosis (Dhar et al 1974) in the epileptic patient. In some instances, oncogenic and

teratogenic activities have been found to coexist in the same individual exposed to hydantoin in utero (Cohen 1981). Adrenal carcinomas, neuroblastomas and ganglioblastomas have also been found to occur (Seelers et al 1979).

3.34 CARCINOGENESIS AND TERATOGENICITY

In the offspring of pregnant animals which have been exposed to certain carcinogenic agents, there are recognised instances of increased tumour incidence, the apparent result of the agent which passed through the placental barrier (Dipaolo and Kotin 1966).

The conversion of a fetal cell to a cancer cell can be thought of as an alteration in its state of differentiation with the ' freeing ' of a programmed stem cell from its normal restraints so that it can form an invasive clone — i.e. become a cancer cell (Cairns 1979). Arene oxides have been found to elicit transformation in cultured cells (Jerina and Daly 1974).

3.4 ' SECONDARY EVENTS ' RESULTING FROM EXPOSURE TO A TERATOGENIC AGENT

3.41 CELL PROLIFERATION, DEATH, SIZE AND INTERACTIONS

Mesenchymal proliferation and mitosis are active in the fetus at the tips of the palatial shelves and DPH may decrease such fibroblastic proliferation, on the other hand, DPH has been reported to increase fibroblastic growth of cells in culture (Paulson and Paulson 1981).

Many important aspects of brain development such as proliferation and cellular migration, differentiation, synaptogenesis and myelination occur in late gestation and postnatally. By manipulating the time at which drugs interfere with cell proliferation, it has been possible to produce selective reductions in specific cell populations in the CNS, resulting in particular types of motor deficit or behavioural change, depending on the structures damaged. In some cases, behavioural effects have been shown after loss of as little as 5% of the total cell population in one area (Barlow 1982).

In relation to DPH toxicity, Livingston (1957) reported persistent ataxia in patients treated with phenytoin and studies have been made describing the loss of Purkinje cells in the cerebellum of epileptic patients having ataxia from high doses of DPH (Hofmann 1958, and Haberland 1962). Similar histological changes were observed by Utterback (1958) and Kokenge et al (1965), following the administration of DPH to cats.

There has been wide support for the stimulatory effect of DPH on connective tissue (Bhussry and Raos 1963, and Houck 1960). Although studies by Robinson (1962) have indicated that stimulation of epithelial elements also occurs. This suggests that a teratogenic influence is being exerted on the lip structures as well as on primary and secondary palatal development (Massey 1966), and cleft palate.

Programmed cell death is as crucial as mesenchymal proliferation since the apposition of the epithelial membranes must be associated with a fusion of the mesodermal elements and loss of the epithelium. Therefore a failure of programmed cell death may be responsible for cleft palate resulting from DPH exposure (Paulson and Paulson 1981).

A recent study (Sulik et al 1980) investigated the pathogenesis of cleft lip and palate and limb malformations using light microscopy (LM) and electron microscopy (EM). At LM and EM, no evidence of cell death in tissues of treated embryos was observed. However, scanning EM studies of the developing primary palate and maxillary process and medial and lateral nasal processes showed a marked reduction in size. At this stage in the control embryos, these three processes normally approximate each other and fuse. In the DPH - treated embryos, cleft lip and palate almost invariably resulted when this approximation is retarded by a reduction in process size, especially in the nasal processes.

Cell morphology, cell differentiation and cell migration (Sulik et al 1980) observations in addition showed a change in the morphology of a subepithelial mesenchymal cell population. A similar cell population exhibits the same alterations in limb buds of embryos exposed to DPH. In both instances, numerous long cell processes, which were found to extend from these cells to form a " cell process meshwork (CPM) " in control embryos, were absent or much reduced in size and number in the treated embryos. A notable change in morphology of the CPM, that is, an increase in complexity with advancing development, has been observed in the control embryos with the processes getting longer and more numerous; this was preventable with DPH exposure.

It was found (Sulik et al 1980) that the critical exposure period for DPH teratogenicity relates to interference with a common developmental phenomenom (CPM formation) and that a specific stage in the development of the CPM is directly related to a critical period of susceptibility to DPH. A substantial role for the CPM in some aspects of morphogenesis is suggested by its morphology and location and by the fact that a disturbance of its integrity is associated with malformation.

With reference to nerve tissue, autopsy findings of a twenty three month old child exposed in utero to anticonvulsant therapy; results showed effects on the cerebellum, where malformations of the dentate nuclei, neuronal heterotopias and abnormalities of the Purkinje cell dendrites were found (Mallow et al 1980). These results suggest a disturbance

of the migration and development of cerebellar neurones. It should be noted that McCredie and McLeod in 1974 reported that there was a neurotoxic action by the well known teratogen thalidomide on the rabbit embryonic dorsal root ganglia, causing the arrest of neuronal maturation as shown by histological examination.

3.42 REDUCED BIOSYNTHESIS

DNA is found in the nuclei of cells and in mitochondria and is synthesized during the S or synthetic phase of the cell cycle. In DNA synthesis, thymine (a pyrimidine)combines with deoxyribose to form thymidine, a nucleoside. Combination of the nucleoside with a phosphoric acid residue leads to the formation of a mononucleotide. Many mononucleotides derived from various purine and pyrimidine bases, combine to form polynucleotides and eventually DNA. The genetic message is coded by the sequence of the purine and pyrimidine bases,in DNA and this message regulates the synthesis of all proteins formed by the cell (Ganong 1975).

The rapidly dividing embryonic cells at the stage of organogenesis have high rates of DNA synthesis and consequently protein synthesis (Köhler et al 1972). Any interference with the base sequence at this stage by a teratogenic agent may cause greater damage and result in altered rates of DNA synthesis. Hydroxyurea and cytosine arabinoside, both known teratogens, have been demonstrated to decrease nucleic acid synthesis in rodent embryos (Kochhar et al 1978, and Krowke and Bochert 1975).

3.5 DPH (PARENT COMPOUND) AND FOLATE ANTAGONISM

Reduced biosynthesis, demonstrated by decreased DNA synthesis measurements would also be the predicted result of DPH toxicity when considering the theory that DPH (parent compound) acts as a folate antagonist in causing a teratogenic effect (see Section 4.2).

DPH is a folate antagonist which may produce its teratogenic effect by causing folate deficiency or rather by inhibiting the conversion of folate to its active derivatives. Folate, the metabolically active form of folinic acid, is an important cofactor for many metabolic processes such as nucleoprotein synthesis and mitosis. If folate deficiency has an aetiological role in DPH teratogenicity, it may be related to the fact that folate is a cofactor for drug hydroxylation reactions, including the metabolism of anticonvulsants (Sullivan and McElhatton 1975). Folate deficiency could lead to a decrease in the rate of metabolism of DPH and thus to higher blood concentrations of the parent compound and perhaps an increase in toxicity. On the other hand, high serum folate concentrations could lead to rapid inactivation of the drug and a subsequent decrease in fit control which might itself have a teratogenic effect (Sullivan and McElhatton 1975).

Animal studies have shown that both folate deficiency and DPH increase the rate of fetal malformations. In general, women taking anticonvulsants who have delivered malformed infants have been shown not to differ from women with normal infants in serum folate levels (Annegers 1974, and Hall 1972). It was concluded that folate deficiency alone is unlikely to be a

cause of fetal malformations. The suggestion that anticonvulsants produce their effect via folate deficiency and that the offspring might be protected from this by folate supplements is entirely unproven (Dukes 1980).

3.6 OTHER THEORIES OF DPH TERATOGENICITY

DPH administered to mice caused embryonic folate antagonism and was found to inhibit ornithine decarboxylase (ODC) the rate-limiting enzyme in putrescine biosynthesis, so causing reduced levels of this precursor diamine aswell as the resultant polyamines, spermidines and spermines. Because ODC is an enzyme of major importance in embryogenesis, any alterations in ODC activity may indicate abnormal development (Paker and Netzloff 1982). Other theories on the mechanism of DPH teratogenicity include altered cortisone metabolism, interference with oxidative metabolism, defects in collagen formation and effects on growth inducers in embryonic life (Wilson 1978, and Paulson 1979).

3.7 SUMMARY

Although it is still not certain whether or not DPH produces a possible teratogenic effect by acting through the parent compound or toxic metabolite (reactive intermediate or final metabolite), evaluation of the evidence seems to suggest that the arene oxide is the toxic agent, especially when considering the information on epoxides per se.

The mechanism of DPH teratogenesis at the molecular and cellular levels may be elucidated by the examination of the effects grouped as ' initial ' and ' secondary ' events. The former which occurs at the molecular level may be examined by a variety of methods. The one used in this study involved the chromosomal method of analysis (Conventional and SCE) which has been shown to be of value in detecting mutational insults.

A chemical mutagen may cause mutations by affecting the nucleotide sequence of DNA molecule in a variety of ways. In the case of fetal tissues which are somatic cells, the mutation would lead to non heritable defects.

The arene oxides have been shown to be mutagenic to mammalian cell cultures, drosophila, bacteriophages and Salmonella typhimurium. A mutation in fetal cells could lead to a change in the structure (sequence) of DNA and a consequent alteration in protein synthesis which could affect enzyme activity and lead to changes in cell growth, proliferation and differentiation resulting in congenital defects.

It has been suggested that some CNS malformations are related to specific gene rearrangements associated with the human equivalent of the mouse T locus which appears to contain an important centre controlling the steps of early embryogenesis, with the genes specifying cell surface components required for cellular interactions at precise stages of development. The T locus is thought to be related to the F9 antigen present on the membrane of early mouse embryos and spermatozoa but

not on adult tissue — a similar distribution to that of human cells.

The covalent binding of the bioactivated intermediate (arene oxide) would provide a molecular basis for carcinogenicity, with a mutation involved in their initiation and cancers have been found in epileptic patients and their offspring. Tumour incidence has been shown to be increased in the offspring of animals exposed to carcingogenic agents and cancer cell transformation of fetal cells has been shown to be elicited by arene oxides in culture.

Deactivation of the arene oxide appears to be the major factor in decreasing DPH teratogenicity. The acitivity of the enzymes involved is genetically determined and their activation and deactivation respond separately to environmental factors. There is varying sensitivity dependent on genotype, maternal exposure to other drugs and to environmental pollutants.

DPH via a possible mutagenic effect may cause the following ' secondary ' events; altered rates of cell proliferation, reduced cell size, altered cell morphology, effects on cell migration and effects on cell differentiation. These events at the cellular level can be monitored by direct morphological observation and DPH has been shown to cause both an increase and a decrease in fibroblastic proliferation in cell culture. DPH has been shown to cause decreased cellular proliferation in the CNS and ataxia due to loss of purkinje cells

in the cerebellum of epileptic patients. Stimulatory effects by DPH on both connective tissue and epithelial tissue was demonstrated and this could possibly be the basis for the manifestation of cleft palate in the FHS.

Programmed cell death and mesenchymal proliferation are both very important in normal development (such as palatial) and DPH can affect approximation and fusion of processes (palatial, maxillary, medial and lateral nasal) due to overall reduction in cell size. Changes in cell process extension of the " cell process meshwork " of mouse limb buds was the result of exposure to DPH and prevented the increase complexity associated with normal development, resulting in malformations. Examination of nervous tissue at autopsy after anticonvulsant exposure in utero has revealed malformation in the dentate nuclei of the cerebellum, neuronal heterotopias , and disturbed migration and development of cerebellar neurons. Histological examination has revealed neurotoxic action by thalidomide, which is a definite human teratogen causing limb reductions.

The study of reduced biosynthesis was investigated by the examination of the DNA synthesis rates for cell populations. A mutagenic agent may alter the base sequence of DNA during the period of organogenesis leading to decreased rates of DNA synthesis. If DNA synthesis is sufficiently inhibited to cause selective killing of the cells and an inhibition of proliferation, these prenatal influences may lead to abnormal growth and development.

CHAPTER 4.

DETECTION OF THE ' INITIAL ' AND ' SECONDARY ' EFFECTS OF A TERATOGENIC AGENT

4.1 DETECTION OF A MUTATION ('INITIAL EVENT')

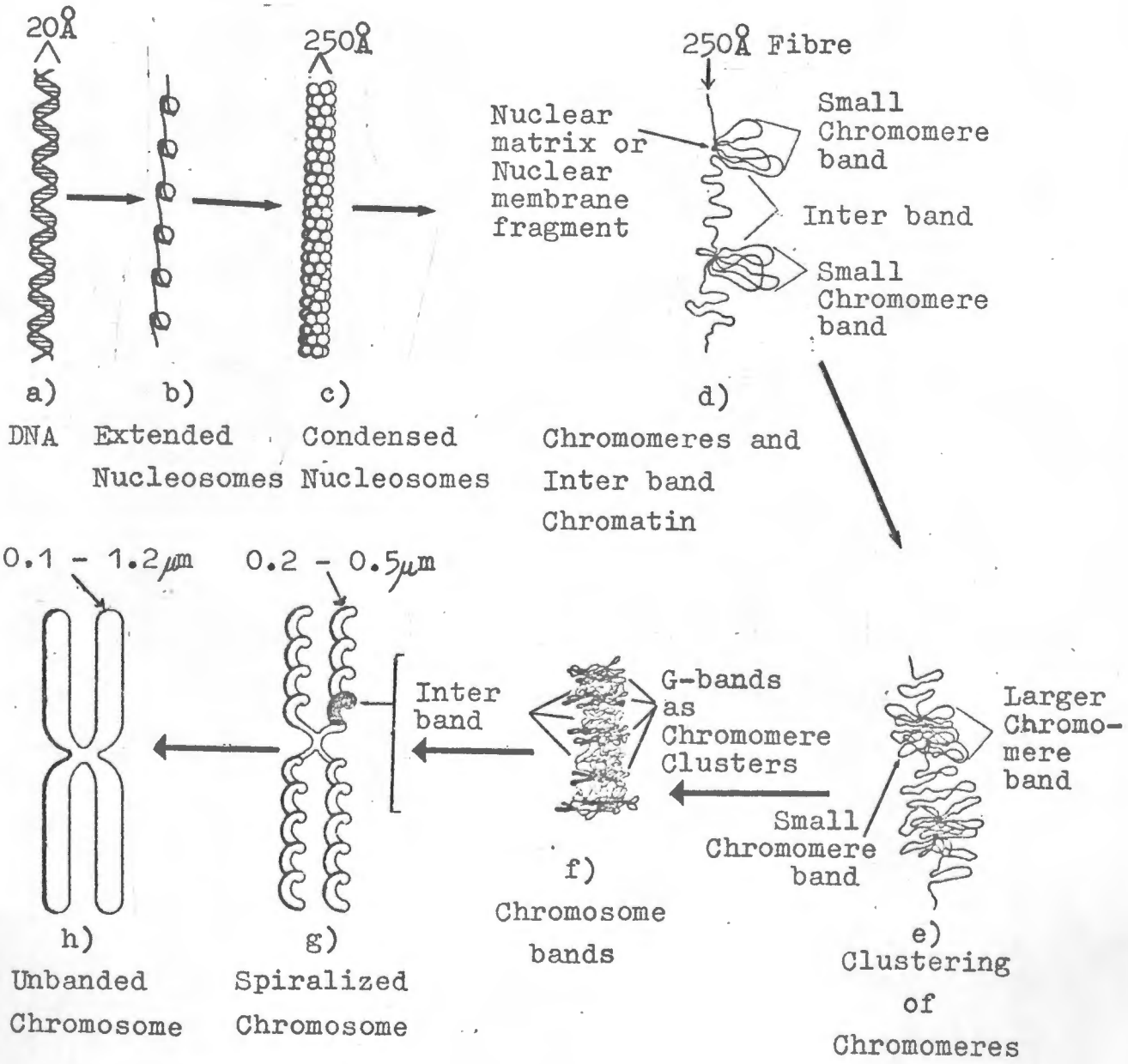
Considerable attention has been focused on the development of new techniques for detecting potential mutagenic substances involving the measurement of DNA damage (see Figure 3 .) Screening tests have been developed which utilize bacteria, yeast and Drosophila as well as other lower organisms. Although these tests have been highly successful in detecting agents which are mutagenic and/or carcinogenic in higher organisms, it is vital to develop complementary systems for detecting agents that may be mutagenic only in mammalian cells (Nakanishi and Schneider 1979). Cytogenetic methods have therefore come to play an important part in mutagenicity detection. As means to examine mutation inductivity and teratogenicity, these methods using chromosome abnormalities as an index are considered a useful screening test (Matsushima et al 1981).

4.12 DNA AND CHROMOSOMES

The structure of mammalian chromatin is based upon a repeating unit called the nucleosome which is comprised of 200 base pairs of DNA coupled with one pair each of the four types of histones, H2 A, H2 B, H3 and H4. Some nucleosomes contain another histone molecule H1. The ' core ' of the nucleosome is

complexed with about 140 highly conserved DNA base pairs and a ' linker ' DNA segment of variable length. During differentiation or cellular specialization, some portions of DNA are permanently inactivated. Certain segments of DNA may be temporarily utilized more extensively during transcription. There is clearly strong biologic pressure for maintenance of the structural integrity of chromosomes in which the DNA resides (see Figure 4.).

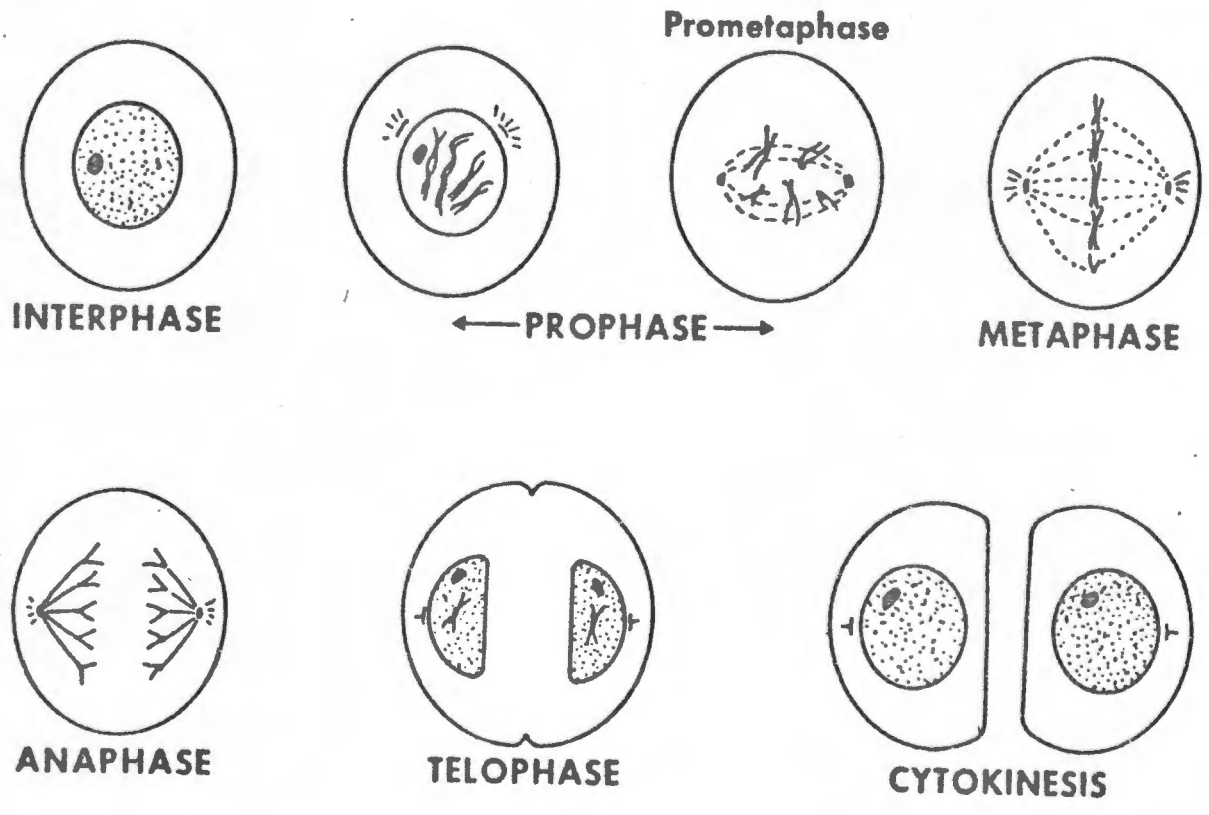
Figure 4. HYPOTHETICAL RELATIONSHIP BETWEEN DNA, NUCLEOSOMES, CHROMOMERES, G-BANDS, AND SPIRILIZED CHROMOSOMES. (Shaeffer Hack and Lawce 1980)



At certain times in the cell cycle and during differentiation, structural changes in chromosomes are correlated with post-synthetic modifications of histones, particularly phosphorylation of H1 and acetylation of H3 and H4, the former probably controls the packaging of coils of DNA during the cell cycle.

In cell division (Figure 5 .), the chromatin becomes condensed into deeply-staining rod-like structures called chromosomes (chromo = stain, soma = body) which are present in specific numbers in each species. This process of forming chromosomes during division is necessary for the parcelling of chromatin into daughter cells.

Figure 5. CELL DIVISION (Shaeffer Hack and Lawce 1980)



The mitotic chromosome as seen by the light microscope consists of two strands called sister chromatids which are held together at a light-staining region called the centromere. The areas above and below the centromere are called the arms of the chromosomes. After cell division, the chromatids are again referred to as chromosomes, having separated from their sisters during anaphase. Chromosomes come in pairs,called homologues, one maternal and the other paternal in orgin. They appear in several shapes depending on where the centromere is located. If the centromere is at the end of the chromosome, the chromosome is called telocentric. This shape is found in the mouse — the entire complement of chromosomes in mice is telocentric. If the centromere is just below the end of the chromosome, it is called acrocentric. If the centromere is not located at the centre or the end of the chromosome, it is called submetacentric. When the centromere is directly in the centre of the chromosome, it is called metacentric. The term acentric is used for chromosomes with no centromere. In addition to the centromere or primary constriction, some chromosomes contain other regions which appear constricted and lightly-stained called secondary constrictions. They have been shown to be the nucleolus organizing area of the chromosome. Some chromosomes have thin stalks above the centromere which usually ends in knobs called satellites. These stalks are actually secondary constrictions (Shaeffer Hack and Lawce 1980).

4.13 CHROMOSOME ABNORMALITIES AND CONGENITAL MALFORMATIONS

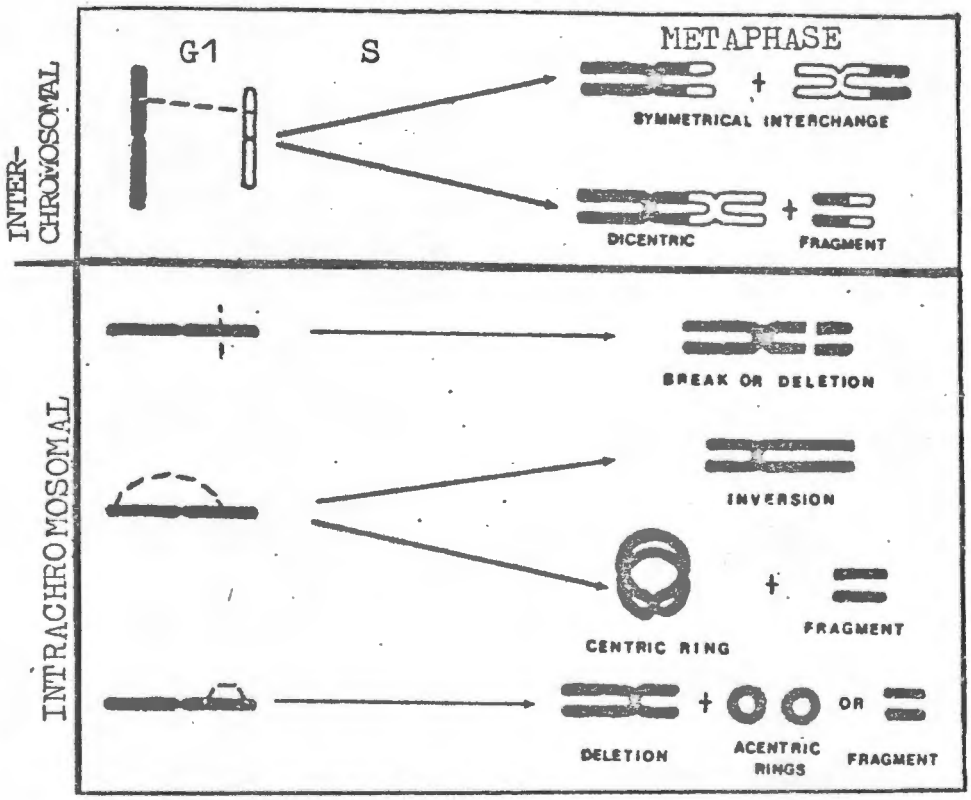
The contribution of gross chromosomal anomalies to human genetic disease burden has been well documented (Jacob 1972). Aneuploidy and structural chromosome anomalies have been shown by various estimates and surveys to have a very significant impact on human fetal wastage as well as on congenitally defective live births (Holden 1982). In humans, it is estimated that approximately 50% of all conception products have a gross chromosome aberration (Carr 1971). Many of these are so severely abnormal that they fail to implant and are thus lost without the mother realizing she was pregnant. However, the great majority of chromosomally defective conceptions are lost during pregnancy as a result of spontaneous abortion (Carr 1967). Even so, a significant number of fetuses with chromosome abnormalities survive to term and beyond. Extensive surveys have set this incidence at one per 200 live births (Lubs and Ruddle 1970). All are associated with marked phenotypic disabilities ranging from relatively mild in XYY, Down's and Klinefelter's syndromes to severe in Pateau's and Edward's syndrome (Yunis 1974).

Recongnition has recently been given to the possibility that many of these alterations may be due to exposure to genetically active agents, and this present study examines this possibility for DPH and its metabolic products.

4.14 ANTICONVULANT DRUGS AND CHROMOSOME ABERRATIONS

Analysis of chromosome aberrations has been the most commonly used method of detecting DNA damage in mammalian cells (Shaw 1970). For a diagrammatic illustration of the formation of chromosome aberrations see Figure 6.

Figure 6. THE FORMATION OF HUMAN CHROMOSOME ABERRATIONS



As many, if not all, chromosome breaking agents are teratogenic, and as these properties have been connected with each other, there has for some time been interest in the study of the cytogenetic effects of DPH (Alving 1977). Conflicting reports about the cytogenetic effect of DPH have appeared in the literature and have been tabulated as follows :-

Table 7 . ANTICONVULSANT DRUGS AND CHROMOSOME ABERRATIONS — POSITIVE RESULTS

SPECIES (Author)	EXPERIMENTAL DETAILS	RESULT
Human (Muniz et al 1969)	DPH added to human lymphocytes in cell culture. Intoxication dose of DPH.	' Chromosome aberrations' found. Exchange-type of abnormalities.
Human (Goodman et al 1969)	Studied cultured leukocytes from children born to epileptic mothers. Results observed probably caused by direct effects of the anticonvulsant drugs on the fetal haemato-poietic system.	' Chromosome aberrations' found.
Human (Márquez-Monter et al 1970)	Blood samples from users of anticonvulsant drugs, and cultured lymphocytes.	Tetraploidy a common abnormality and hypotetraploidy
Human (Herha and Obe 1977)	Lymphocyte cultures of blood taken from treated epileptics.	Exchange-type abnormalities

Continued/...

Table 7. Continued/...

SPECIES (Author)	EXPERIMENTAL DETAILS	RESULT
Human (Neuhaüser et al 1970)	The chromosomes of epileptic mothers and their children were analysed using lymphocyte cultures.	A high-level of chromosome defects was detected in both cases. No congenital defects recorded.
Rat (Roman and Caratzali 1971)	The authors examined bone marrow cells of rats treated with DPH.	There was a high-level of abnormal metaphases. This anomaly recovered to the normal level seven days after withdrawal of DPH.
Human (Grosse et al 1972)	The chromosomes of 32 women suffering from epilepsy and treated with anticonvulsant drugs were examined, and those of their children (aged 0 - 3 years) who had been exposed to the drugs during pregnancy.	Chromosome aberrations were found. The results were highly significant when compared with un-treated controls. A significant correlation was found between the aberration rate of mother and child. No congenital defects observed.

Table 8 . ANTICONVULSANT DRUGS AND CHROMOSOME ABERRATIONS — NEGATIVE RESULTS

SPECIES (Author)	EXPERIMENTAL DETAILS	RESULT
Human (Muniz et al 1969)	The chromosomes of lymphocytes taken from seven patients with epilepsy and treated with DPH were examined and found to be normal.	There were no chromosome abnormalities in lymphocytes after in vivo exposure to DPH.
Human (Brøgger 1970)	The chromosomes of lymphocytes from epileptic patients receiving therapeutic doses of DPH were found to be normal.	The therapeutic dose of DPH does not cause chromosome abnormalities in lymphocytes in vivo.
Human (Bishun et al 1975)	The authors investigated the chromosome breaking effects of various anticonvulsants (including DPH) in peripheral blood lymphocyte cultures and found that there were no apparent chromosome defects (breaks, rearrangements, etc.) produced by these drugs at the concentrations used (DPH 10 to 70 μg/ml), although mitosis was inhibited at concentrations above these.	Exposure of peripheral blood lymphocytes in vitro to DPH and other anticonvulsants did not cause the production of chromosome abnormalities. However, the authors make the important point that metabolites of DPH produced in vivo and not in culture, may be mutagenic.

Continued/...

Table 8. Continued/...

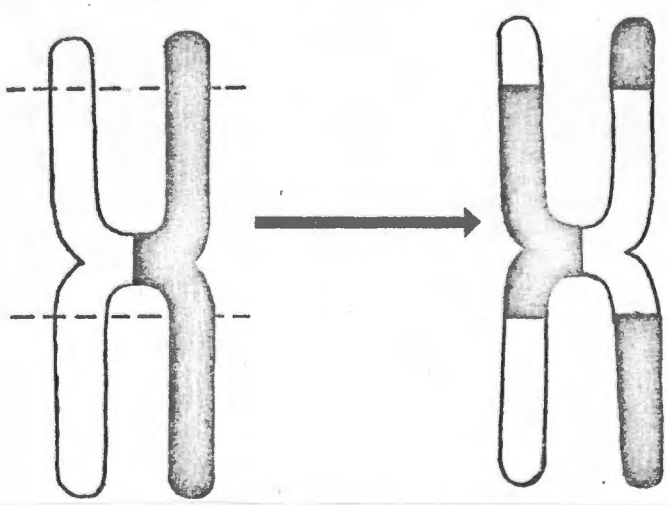
SPECIES (Author)	EXPERIMENTAL DETAILS	RESULT
Human (Alving et al 1976)	The authors were unable to confirm the results found by Muniz et al (1969) using human lymphocytes with DPH added in cell culture.	No chromosome abnormalities were found.
Human (Alving et al 1977)	Peripheral blood lymphocytes from ten patients with epilepsy and their bone marrow cells were examined for possible chromosome abnormalities resulting from in vivo treatment with DPH.	There was no significant evidence of chromosome damage in either samples.

4.15 SISTER-CHROMATID EXCHANGE

Sister-chromatid exhchanges (SCEs) have been utilized to detect DNA damage in eukaryotic cells (Abe and Sasaki 1977; Kato 1974b; Latt 1974 ; Perry and Evans 1975; Popescu et al 1977; Stetka and Wolff 1976 , and Wolff et al 1977).

A SCE is the cytological manifestation of an event occurring at the DNA level; its formation is dependent upon a four-stranded exchange. The exchange can be observed cytologically in metaphase chromosomes (Figure 7 .) following two cycles of DNA replication in the presence of the thymidine analogue 5 - bromodeoxyuridine (BrdU) (Carrano 1980). These events were first observed by Taylor et al (1957) with tritiated thymidine labelling and autoradiography. A SCE is derived from the breakage and the repair of chromosome fibres, which is now generally considered to concern the DNA molecule responsible for the linear continuity of the chromosome (Figure 7.).

Figure 7. FORMATION OF SISTER CHROMATID EXCHANGES



CORRECTION
The interchange of a complete interstitial "block" of chromatid within a replicated chromosome requires 2, not 1, SCEs. Although this alters the basal level of SCEs stated and the SCE results, since all treatments were analysed in the same way, the observation of no difference between control and treatments remains valid with DPH causing no increase in the number of SCEs detected.

These aberrations are the result of misrepair of DNA, the chromosome being considered as the visualized DNA molecule (Brøgger 1979). The initiation of DNA damage in proliferating cells by a chemical agent is likely to delay DNA replication and may lead to post replication repair and possibly mutagenic errors in repair (misrepair). A high rate of cell turnover will be more susceptible to the effects of chemical exposure than non-dividing tissues due to insufficient time for repair and a higher frequency of persistent DNA damage. In theory, DNA repair should impose a threshold on the dose-effect relationship for a given agent, that is, provide an absolute defence mechanism if all the DNA damage induced by that agent was repaired by an error-free process, but this is not the case. Therefore, the property of an agent to stimulate DNA repair in a test system, observed as SCE s , should be taken as an indication of its ability to induce DNA damage (Lambert and Harper 1979).

In mammalian cells, a SCE rate of 7 - 18 per cell seems to be the normal range with moderate amounts of BrdU (Latt 1981; Kato 1974b; Wolff and Perry 1974; Wolff et al 1975; Galloway and Evans 1975; Latt 1974b; Latt et al 1975; Tice et al 1975, and Changanti et al 1974).

If SCE s are used to assess in vivo or in vitro chromosome damage by drugs, then information on the basal level of SCE$_s$ in the cells, that is, without drug treatment, must be obtained. The possible sources of variation due to external factors affecting the SCE rate would also have to be assessed.

4.16 BIOLOGICAL AND PHYSICAL FACTORS AFFECTING THE SCE RATE

Work by Waksvik et al (1981) examined the effects of age, sex, and genetic factors on the SCE process as analysed by the occurrence of SCEs in cultured lymphocytes of human twin pairs. The age difference was statistically significant but not sex difference. The analysis of variance did not reveal any significant differences in the total or within pair variance. It was concluded that genetic variance does not have a major influence on the between-subjects variation in the mean SCE value.

However, an investigation of inherent inter-individual differences, which may be genetically influenced, has been performed in a study of familial patterns of SCE frequencies in complete 2- or 3- generation family units (Cohen 1982). There were significant differences among, but not within, families in the SCE rates. Therefore, a genetic contribution to the SCE frequency was suggested by the observed pattern of familial correlations, in contrast to the report by Waksvik et al (1981).

It is meaningful to refer to the differences in in vivo and in vitro SCE frequencies. The in vitro SCE frequency is generally higher than that in vivo in animals, suggesting that SCEs observed in vitro involve a fraction of SCEs induced by various physical factors such as the components of the media, including sera (Kato and Sandberg 1977) and antibiotics, pH, temperature (Kato 1980), light (Ikushima 1977), BrdU concentration (Kato 1974 a, and Tice et al 1976b), the change of original in vivo cellular characteristics as well as other unknown factors (Tsu and Kato 1981).

4.17 CHEMICAL MUTAGENS AND SCE FORMATION : INITIATING LESIONS

There is no unique initiating lesion that leads to the formation of SCEs, rather their induction appears to arise from a wide spectrum of lesions, with varying efficiencies. With few exceptions, an agent that induces mutation also induces SCEs. This may be due to the sensitivity of mammalian mutagenicity assays for detecting weak mutagens, or may indicate in fact, that the lesions that form a mutation may be a subset of the lesions that form SCEs (Carrano 1981).

Although the exact mechanism of SCE formation is not known, it is well established that the frequency of SCE is increased by exposure of cells in vivo and in vitro to known mutagens (Perry and Evans 1975; Abe and Sasaki 1977; Allen et al 1977, and Stetka and Wolff 1976). Therefore, the SCE index has come into use as a sensitive means of detecting DNA damage and the bioassay has already been used to monitor the exposure of humans to potentially harmful chemicals (Funnes - Cravioto 1977).

4.18 ANTICONVULSANT DRUGS AND SCE FORMATION

Recent studies on the mutagenic potential of anticonvulsant drugs have centred on the analysis of SCE frequency rates and have been tabulated as follows:

Table 9. ANTICONVULSANT DRUGS AND SCE FORMATION

SPECIES (Author)	EXPERIMENTAL DETAILS	RESULT
Human (Hunke and Carpenter 1978)	The frequency of SCEs for human lymphocyte cultures treated in vitro with various concentrations of DPH (10 to 100 μg/ml) was determined.	Positive — The SCE frequency increased with increasing DPH concentration in vitro. A decline in the mitotic index was also noted at higher concentrations.
Human (Hunke and Carpenter 1978)	The SCE frequency was determined for lymphocytes in culture from six control subjects and ten patients undergoing monotherapy with DPH for an average of 2.6 years. Serum DPH levels ranged from 3.8 to 29.5 μg/ml.	Negative — There was no significant difference between the frequency of SCEs of the DPH treated (in vivo) lymphocytes and controls.
Human (Seshadri et al 1982)	These experiments were performed to see whether SCE analysis of mothers and their neonates would detect in utero exposure of the fetus to DNA-damaging agents (including DPH and other anticonvulsants).	Negative — All the infants born to the epileptic mothers were congenitally normal with normal SCE rates.

4.190 THE BIOLOGICAL SIGNIFICANCE OF CHROMOSOME ABERRATIONS AND SCEs

Whatever the nature of the lesions in the chromosome that ultimately lead to the formation of SCEs, because SCE induction differs in very many ways from the induction of ordinary chromosome aberrations, it is likely that the lesions will be quite different from those that lead to aberrations (Wolff 1977). SCEs for instance, are induced at high frequencies by concentrations of chemicals that induce very few aberrations (Latt 1974a; Perry and Evans 1975, and Solomon and Bowbrow 1975), and they are not markedly increased by low doses of ionizing radiations, whereas aberrations are (Perry and Evans1975, and Wolff et al 1974). Cytogenetic analysis in patients following adriamycin treatment (Nevstad 1978) have provided in vivo confirmation of the observation, first made in vitro, that mutagenic agents are more readily detected by SCE analysis than by conventional analysis (Perry and Evans 1975).

SCEs react differently to treatments with caffeine than do chromosome aberrations (Kato 1973, and Kihlman 1975), and they are not correlated in any consistent fashion with the increased aberrations seen in human diseases; Bloom's syndrome (Changanti et al 1974), ataxia telangiectasia (Galloway and Evans 1975) and Fanconi's anaemia (Latt et al 1975), since the SCE levels are very high in Bloom's syndrome, normal in ataxia and reportedly low in Fanconi's anaemia.

It therefore appears that SCEs may be the result of fundamentally different cellular lesions and processes from those that cause

chromosome aberrations (Wolff et al 1977). It has been postulated, that although the majority of SCEs are probably genetically neutral because equal amounts of sister chromatid are exchanged, some unequal exchange could occur leading to deletion, insertion or frameshift mutation (Wolff 1977). Nevertheless, the actual biological significance of SCE remains obscure.

4.191 ADVANTAGES AND DISADVANTAGES OF CHROMOSOME ABERRATION AND SCE ANALYSIS TECHNIQUES

Biologically, aberrations are associated with cell death, whereas SCEs do not seem to be so, thus making it likely that SCEs are more representative of events compatible with cell survival, such as mutagenesis (Wolff 1977).

The methodology for performing the SCE assay is relatively simple, the data quantitative and rapidly collected. Statistically significant results can be obtained when only twenty cells are scored, as contrasted with the tedious lengthy scoring required to observe significant differences with ordinary chromosome aberrations (Wolff and Carrano 1979).

SCE is an extremely sensitive indicator of DNA damage and it is possible to detect SCEs at far lower concentrations of compounds than are needed to produce chromosome aberrations (Latt 1974a; Perry and Evans 1975, and Wolff 1977). However, the SCE assay may be more prone than chromosome aberrations to false positive results due to sensitivity of the actual technique to physical factors. Another

disadvantage lies in the technique itself: by the necessity of adding BrdU, in no case is the activity of the test agent alone measured, but always the co-activity of the test agent and BrdU. Theoretically, this could lead in some cases to the observation of a co-mutagenic rather than mutagenic activity (Gebhart 1981). False negative results may also be apparent with the SCE assay since it is thought that the SCEs may reflect only a very small proportion of the DNA damage that has occurred after the exposure to a DNA damaging agent (Latt et al 1981).

SCE analysis is a method which allows a sensitive investigation of part of the spectrum of activity on DNA by mutagens (Gebhart 1981), although as described, there are some serious arguments against a general application of this method for the screening of mutagens.

4.19.2 WHICH TECHNIQUE SHOULD BE USED TO INVESTIGATE POSSIBLE DPH MUTAGENICITY ?

Several investigators tended to replace the ' painful ' classical cytogenetic analysis of mutagen treated cells with the simple SCE test. The fact that the molecular mechanisms of SCE formation were, and are even now, largely unknown, was apparently not considered as too disturbing. Not enough attention was paid to the fact that most of the steadily increasing number of agents contributing to the high correlation observed between break and SCE induction have the same mechanism of action (i.e. alkylation), thus rendering a high similarity of their cytogenetic effects fairly probable. Chemicals which react with many different sites on DNA structure and/or metabolism, could probably induce both chromosome aberrations and SCEs,

whilst agents with a small and sometimes highly specific spectrum of activity may induce one or the other (Gebhart 1981).

It has been suggested that the SCE technique should be considered as a valuable additional method for cytogenetic mutagenicity testing, which however is not adequate to replace the classical methods of analysis of structural chromosome damage (Gebhart 1981).

4.2 DETECTION OF THE ' SECONDARY EVENT ' OF REDUCED BIOSYNTHESIS : DNA SYNTHESIS

The amount of DNA sythesized by a proliferating cell can be assessed by measuring the amount of a radioactive precursor incorporated into DNA. ^{3}H- thymidine incorporation into DNA would thus be a measure of the amount of newly synthesized DNA and can be assessed by liquid scintillation counting, the results ofwhich can be expressed as disintegrations per minute (dpm) of the incorporated radioactivity.

It has been shown that 0.2 μM/ml concentration of DPH in the culture medium of whole mouse embryos caused a significant reduction in DNA synthesis (Beyers 1981, MSc Med Thesis, UCT). Research by Hassel in 1980 showed that DPH applied for two days to cultured human fibroblasts at concentrations of up to 5 μg/ml did not appear to affect protein, collagen, or DNA synthesis in normal fibroblasts. However, cells obtained from DPH - enlarged gingiva contained higher quantities of DNA, incorporated ^{3}H- leucine into protein more rapidly, and produced a higher percent of collagen. At concentrations of 10 μg/ml or greater, DPH inhibited cell growth and synthesis of protein.

4.21 DETECTION OF THE EFFECTS ON CELLULAR DEVELOPMENT

Much work in this area has been concerned with the direct observation of the whole embryo or animal embryonic tissue using the light microscope or scanning and transmission electron microscopy (Sulik et al 1980), or with the examination of post mortum material by histological techniques (Livingston 1957; Utterback 1958, and Kokenge et al 1965). The effects on cell development included, 1) increased or decreased cell proliferation (mitosis), 2) reduction in cell size, and 3) changes in cell morphology with effects on cell migration and differentiation.

Embryonic cells that undergo differentiation in culture have also proved an appropriate system for detecting teratogenic activity (Wilk et al 1980). Alterations in growth or differentiation of the teratogen-treated chick limb mesenchyme cells and neural crest cells included detachment of the cells from the substratum, decreased cell proliferation and changes in cell morphology with failure to develop into the expected phenotype.

These alterations of cell growth in culture are thought to be similar to those effects on cell development already mentioned (see 3.2) and it is thought that similar events can cause abnormalities in the fetus exposed to teratogens (Wilson 1977). Teratogens acting through these mechanisms would be detected in cell culture and this system might also be effective for screening teratogens that interfere with interactions between cells and matrix, and with migration (Wilk 1980). Using

this cell culture testing model, Wilk (1980) observed that DPH at 50 µg/ml caused 25% chick neural crest detachment when compared with controls, and also caused a large reduction in limb bud mesenchyme cell differentiation which was less severe when DPH was incubated with the S-9 mixture required for metabolic activation, indicating that perhaps the parent compound caused most teratogenicity.

4.3 SUMMARY

Methods for detecting potentially mutagenic substances usually involve measurement of DNA damage. As lower organisms are usually used in mutagenicity tests (viz. bacteria etc.), there needs to be developed systems which detect mutagens which are mutagenic and consequently teratogenic to mammals only. Cytogenetic studies using mammalian cells would seem appropriate. Chromosomes are the highly organised structures in which DNA resides and can be examined microscopically during the metaphase of cell division. There is a huge contribution of gross chromosomal anomalies to fetal wastage and congenital defects which may be due to in utero exposure to genetically active agents. Chromosomal defects which can be detected by light microscopy includes gross chromosomal aberrations (dicentrics, acentrics, breaks, deletions, inversions and ring chromosomes) and sister chromatid exchanges (SCEs).

The mutagenic effect of DPH on human chromosomes has been studied and conflicting reports ensued. In vivo studies have shown positive results with regard to the examination of leukocytes taken from the children of epileptic mothers who had

been administered DPH during their pregnancies. No increase in the incidence of congenital defects was observed. The mothers also displayed chromosomal aberrations including exchange-type defects and a high incidence of tetraploidy was found to occur. On the other hand, investigators have shown no chromosome aberrations to occur in the leukocytes or bone marrow cells of epileptic patients treated with DPH in vivo. However, rat bone marrow cell analysis has shown a high level of abnormal metaphases due to in vivo exposure to DPH with recovery on withdrawal of the drug. In vitro exposure of human leukocyte cultures resulted in exchange-type chromosome aberrations in one instance but negative results in another, which may be due to the lack of; metabolic activators in the medium during cell culture, or prior metabolic activation of the drug.

The ability of an agent to cause DNA damage and stimulate repair in a test system has been estimated with the investigation of SCEs (the result of misrepair). In mammalian cells, the normal basal SCE rate is 7-18 per cell with variation due to external physical factors during cell culture having some effect, giving a generally higher rate for in vitro exposure in comparison with in vivo drug exposure. With few exceptions, an agent that induces a mutation also induces SCEs although the exact mechanism is unknown. The technique would provide a sensitive means of detecting DNA damage with low drug concentrations and only 20 cells needs to be scored per treatment in order to obtain statistically significant results.

The in vitro exposure of human lymphocytes to DPH has been found to result in an increased SCE rate and a decrease in the mitotic index however, the examination of the leukocytes from epileptic mothers and their children exposed to DPH in vivo has shown no increase in the frequency of SCEs from normal and there was no increase in the incidence of congenital malformations. If DPH acts on many different sites of DNA structure, it could possibly induce both chromosome aberrations and SCEs which probably result from fundamentally different processes. If there is highly specific activity, the agent may induce only one or the other and so an examination of both is necessary. Mutagenic agents are thought to be more readily detected by SCE analysis than by conventional chromosome analysis which tends to lead to cell death whilst SCEs are compatable with cell survival.

Detection of reduced biosynthesis (DNA) has been made possible by measuring the amount of DNA precursor (^{3}H-Thymidine) incorporated into DNA. DPH has been shown to cause a significantly decreased rate of DNA synthesis in whole mouse embryos exposed to DPH in vitro. In humans with enlarged gingiva, DPH has been shown to cause a decreased rate of DNA synthesis and consequently reduced protein synthesis and cell growth, although the drug has been shown not to affect the DNA synthesis rates of human fibroblasts in cell culture.

Examination of DPH affects on whole embryos, embryonic tissue and post mortem material has shown changes from normal in the cell proliferation rates, size, morphology and differentiation. Embryonic cells undergoing differentiation in culture, such as

chick limb mesenchyme cells and chick neural crest cells has led to cell detachment and all of the other changes mentioned above which could eventually lead to congenital malformations.

CHAPTER 5.

THE MONITORING OF CONGENITAL ABNORMALITIES

5.11 TERATOGENICITY TESTING AND SCREENING METHODS: HUMAN STUDIES

The use of pregnant women to test agents directly for teratogenicity is fraught with moral and ethical implications that make such studies impossible to perform (Chenoff and Lyons Jones 1981). The study of prenatal losses however may provide a sensitive index of the response to a teratogenic agent (Shepard 1979). The 3% of neonates with congenital defects represents only the tip of the iceberg when compared to the total number of embryonic/fetal losses. By studying embryos and fetuses from spontaneous abortions in the first trimester, an earlier indication of the trouble might be noted, which could provide epidemiologic information some six months before a teratogenic effect would otherwise be detected in newborns. Another advantage of this early monitoring is a short period from the teratogenic exposure to the interview and examination of the mother. This could be less than a week as compared to the seven to eight month interval when the history is taken from the mother of a neonate. There may however be some difficulties in obtaining an unbiased account of the case history.

Monitoring facilities for defects in neonates exist in many countries but these facilities probably fail to detect minor changes in brain function or long-term carcinogenesis.

Generally, only easily recognized physical defects are recorded; the larger portion of congenital disease (60%) is identified after the newborn period and so is not included, also artifacts may be associated with data collection. However, a continuous recording and registry of congenital defects could perhaps provide an important warning of teratogenic action by a new drug (Shepard 1979).

The majority of attempts to identify teratogens in human beings have however been retrospective epidemiologic studies on the malformed child which has very serious flaws when applied to teratogen identification. For example, it seems to concentrate on the easily recognizable malformations, whilst the patterns of less obvious malformations may never be identified. These studies also rely heavily on maternal history which may have been inaccurate. While epidemiologic studies have been successful in identifying ' epidemics ' of particular major malformations, such as limb reductions, they generally (an exception is thalidomide) have not been successful in identifying any probable cause for these ' epidemics ' (Chernoff and Lyons Jones 1981).

The monitoring of human populations is a retrospective study — the damage has been done. The most important thing would be to prevent a drug from ever being allowed to be administered to the pregnant woman (except in extreme exigency) and causing damage to the unborn child. Therefore, teratogenicity testing should be the first step in the line of defence and monitoring the second (Shepard 1979).

In contrast to human population studies, inwhich extremely large numbers of pregnancies must be analysed to provide statistically significant data relative to the teratogenicity of a drug, appropriate animal model systems which approximate the human situation can readily provide valuable information. Unlike human studies, variables can be well controlled and the effects of the drug more easily delineated (Sulik et al 1980).

5.12 TERATOGENICITY TESTING AND SCREENING METHODS: ANIMAL STUDIES

The U.S. Pharmaceutical Manufacturers Association established in 1962 founded a Subcommittee on Teratology which was involved in activities which culminated in the publication in 1966 of " The Guidelines for Safety Evaluation of Drugs for Human Use ", more familiarly referred to a the " FDA Guidelines of 1966 ". Modest extensions and changes have been made, but still the FDA guidelines of 1966 remain as the prototype for teratological testing procedures (Wilson 1979a).

The standard teratogenicity testing in pregnant laboratory animals was created only after the devastating effects of thalidomide in the early 1960's (Shepard 1979). The FDA instituted legislation which required that no drug for human consumption, which may be administered to pregnant women, will be released for sale without first having been tested in at least three non-human animal species, that is, at least two rodents and one other of the dog, rabbit, or cat (Wilson 1979a).

Recommendations given for testing drugs during the reproductive cycle of animals were the conventional six week chronic toxicity tests in male and female animals; these were followed over two pregnancies, and fetal survival was the main parameter measured in this so-called ' litter - test ' (Schardein 1976). However, this test was criticized since the test could keep valuable and teratogenically innocuous compounds (in humans) off the market while permitting teratogens to reach the public (Wilson 1979).

Besides being time consuming, inefficient and costly, animal studies give no guarantee that a given agent, tested in the legal minimum of animals and found free of side effects, will be equally innocuous in humans. Although animal studies have played a major role in elucidating the principles and mechanisms of teratogenesis, they have not been successful in identifying human teratogens (Chernoff and Lyons Jones 1981). The reason for this failure can be found in the principle of teratology that states that susceptibility to teratogens depends on the genotype (strain and species) of the conceptus (Wilson 1973). Therefore, an important question arises as to which species should be used for these tests.

While the only true model for human DPH - induced teratology is clearly the human, other animal species, particularly the mouse may be valuable for investigating the teratogenic effects of DPH and elucidating the possible mechanisms involved.

5.2 THE FETAL HYDANTOIN SYNDROME IN THE MOUSE

A valid animal model of the Fetal Hydantoin Syndrome (FHS) has been developed called the " Quaker " mouse model (Finnel 1977). Mouse neurological mutant quaking is a mouse strain predisposed to spontaneous seizures. The model was designed to assess the overall teratogenicity of DPH, with the resulting malformations duplicating many of the features of the FHS as it appears in man. It helps to confirm the aetiologic role of DPH rather than maternal seizures as a cause of congenital malformations and shows that mice are susceptible to its teratogenic effects. DPH is also clearly teratogenic both in A/Jax mice, a strain spontaneously susceptible to fetal anomalies (Massey 1966) and in Swiss-Webster mice (Harbison and Becker 1969). This illustrates the important point that experimental results in the mouse may have validity when extrapolated to man — perhaps even more so than the much more costly and elaborate methods using subhuman primates, since in the monkey only slight teratogenic effects of DPH have been observed (Nakan 1980).

5.31 IN VIVO ANIMAL TERATOGENICITY TESTING : ADVANTAGES AND DISADVANTAGES

In vivo teratogenicity testing in trinsically allows for the activation of those drugs which cause their effects by a product of their metabolism and also allows for all other factors which may affect the actual dose of the agent, including maternal metabolism and placental barriers. However, an inherent problem of animal in vivo testing of teratogenic agents on the pregnant female and the later examination of the fetus

for possible pathological effects is that there is little control over what precise form the applied drug is being presented to the fetus, and in what concentration it is being delivered. The pharmacokinetics of the experiment cannot be fully defined and the pharmacodynamics of a teratological effect are also difficult to establish (Williams 1982).

Futhermore, these experiments are time consuming and expensive since currently approved tests on drugs for possible teratogenic effects on the human fetus generally include the examination for morphopathology and for histopathology of a large number of rodent fetuses, at various ages, obtained from pregnant females injected or fed with the substance in question, at different stages of pregnancy and at different dose levels (Williams 1982).

5.32 IN VITRO ANIMAL TERATOGENICITY TESTING: ADVANTAGES AND DISADVANTAGES

The use of in vitro methods and organ culture techniques using differentiating embryonic tissues in particular, allows for the investigation of the embryotoxic potential of an agent while excluding the complicating pharmacokinetic factors. It makes possible the study of the direct effect of the original agent or its defined metabolites on the developing and differentiating system (Barrach 1978).

Whole explanted embryos, organs or their parts in culture require many growth measurements, histological and biochemical investigations which are time consuming. Cell culture-based assays however, can assess the effect of a teratogenic substance

on differentiation in a relatively short time. Cell cultures are generally more homogeneous and so can be exposed to the possible teratogenic agent in a more controlled fashion. These cultures are also more suited to biochemical and chromosomal assays, although cell-cell recognition properties. histiotypic assembly and cell replication may also be measured accurately. Using ultrastructure techniques, neurotoxic effects may also be studied by assessing the effect of a suspected substance on neural outgrowths and synaptic connections (Clayton and Zehir 1982). The confounding factors of maternal, nutritional and hormonal considerations are excluded. The exact drug concentration and time of exposure and the developmental stage can be defined. In addition, cell cultures are relatively inexpensive and simple to maintain.

A serious limitation of in vitro techniques is that the drug concentration in the culture dish derived directly from the dose per body weight of the mother does not allow for the effects of maternal - fetal partition or for rapid elimination by the mother, which would reduce fetal dose. The more serious problem of whether the drug exerts its teratogenic effect directly or through a toxic or persistent metabolite can be approached by culturing in the presence of an activating system which is capable of the biotransformation of the drug (Ames et al 1975). There is much support for the idea that in vitro testing should play a more important role, along with in vivo experiments, in the campaign against teratogenic agents (Barrach 1978, and Shepard 1979).

Recently, the preparation and properties of whole liver cell cultures have been reported, both as isolated hepatocytes and as cultures of liver slices (Liddiard et al 1978). Their advantages over microsomal preparations include a long life-span, an independence of cofactors and the retention of the total cellular metabolism of the drug. However, it has not been possible to decide which of the in vitro methods is most appropriate for purposes of prediction of teratogenicity (Merker 1978). In the present study, rat liver microsomal fractions were used because they were prepared elsewhere and kindly made available to the author. Other preparations were not available.

Much valuable information about human fetal drug metabolism has been gained by using human fetal liver for metabolic activation (Nau and Neubert 1978), since the human embryonic liver can metabolize a wide variety of xenobiotic substances as early in gestation as during the period of late organogenesis. The use of human fetal liver tissue would allow much better extrapolation of results to the human situation than is possible with rat liver tissue.

5.4 METABOLIC ACTIVATION : LIVER PREPARATIONS

Numerous teratogenic agents may require metabolic activation before their effects may be manifest. In contrast to the in vivo system, activation products cannot be formed by embryonic tissue in vitro (Kittel et al 1978). Therefore, for the assessment of indirect teratogens with mammalian cell cultures, several experimental systems have been developed.

The incubation of chemicals with liver microsomes allows for metabolic activation (Uehleke 1973). Liver microsomes can be obtained using the relatively convenient and simple method of Ames et al 1975. The incubation of DPH with rat liver 9,000 X g supernatant and with rat liver microsomes has demonstrated that NADPH - dependent mixed function oxidases are involved in the production of p-HPPH (Kutt and Verebely 1970, and Kutt and Fouts 1971) the major metabolite in the rat (Chang and Glazko 1972). Hepatic microsomal enzymes are probably involved in the conversion of DPH to the DHD metabolite (Lu et al 1977) and hepatic soluble enzymes are likely to be responsible for the conversion of the DHD metabolite to catechol (Jerina et al 1970a).

The disadvantages of this cell-free method include the changes in the organelles during preparation, the absence of influences caused by other organelles such as mitochondria, the changes in the level of cofactors and the inevitable neglect of permeability factors and metabolic processes taking place in other cellular components.

5.51 CELL CULTURE AND IN VITRO TERATOGENICITY TESTING

Ideally, the study of the effect of a possible teratogenic agent should be performed on differentiating embryonic cells. Mouse limb buds in organ culture have already been tested as indicator organs for identifying teratogenic compounds (Kochhar 1975). The potential of this system was extended to the use of chick limb mesenchyme cells and neural crest cells (Wilk et al 1980; Section 4.21), where the teratogenic effect was judged by alterations in cell morphology.

The rapid teratogen screening method of cell culturing may not detect biochemical changes that occur in the absence of morphological alterations, although these cell cultures would allow easy application of techniques for biochemical investigations such as the analysis of the rate of DNA synthesis in control and treated (drug present) cultures. Therefore, this system would be sensitive to teratogens that specifically alter tissue development and to agents that inhibit overall growth processes. Another disadvantage of this in vitro animal model is that the differentiating cells have to be excised from the developing chick embryo at particular stages of early development, depending on which stage of differentiation is to be studied in vitro (Wilk et al 1980).

Differentiating cells in culture can therefore provide a rapid means of identifying potential teratogens and as such should be be a practical adjunct to animal testing.

The in vitro model of differentiating cells in culture would lend itself well to the application of cytogenetic techniques and the investigation of mutagenic effects. Cells to be used for cytogenetic testing should possess the following qualities: 1) long-term monolayer cell lines that are not fastidious in growth requirements or handling, 2) short cell cycle for easy propagation with high mitotic rate, and 3) favourable chromosome characteristics including low diploid number.

Several murine embryonal carcinoma cell lines seem very similar to early embryonal cells by having the capacity to differentiate in vitro into some derivatives of the three primary germ cell layers (Martin 1975), and for these reasons such cell lines may be of value in studying the teratogenicity of potentially embryotoxic agents. These cells are also suitable for cytogenetic analysis since they possess all of the desired qualities mentioned above. One of these cell lines, PC13, was used in this investigation as the in vitro animal model.

5.52 NORMAL EMBRYONIC CELLS AND EMBRYONAL CARCINOMA CELLS

It is clear that numerous similarities exist between the cells of the early embryo and embryonal carcinoma cells or the stem cells of a teratocarcinoma (Martin 1975). Pluripotency, or the ability to proliferate in the undifferentiated state and to give rise to differentiated somatic cell types, is displayed by both cell types. Differentiation of the embryonal carcinoma cells occurs by a process similar to normal embryogenesis. The fact that endoderm is the first differentiated cell type formed by embryonal carcinoma cells in vitro suggests

that on a functional level, all embryonal carcinoma cells resemble the cells of the inner cell mass of the four day embryo.

Ultrastructurally, they both have the morphology of the undifferentiated cell type. They share biochemical properties such as alkaline phosphatase activity, which is present at high levels. The two cell types have at least one specific surface antigen in common, which has so far not been found in any other cell type except sperm (Martin 1975).

Murine PC13 embryonal carcinoma stem cells or EC cells were used in this investigation and therefore the problem remains of extrapolation of results of any teratogenicity to the human situation.

5.61 MORPHOLOGICAL CHARACTERISTICS OF THE UNDIFFERENTIATED PC13 EMBRYONAL CARCINOMA CELLS

PC13 EC cells are small, Epithelial-like cells that have indistinct cell boundaries and their nucleocytoplasmic ratio is high (Nicholas et al 1976). Their cytoplasm as seen by electron microscopy contains few organelles, some mitochondria, a large number of free ribosomes, little endoplasmic reticulum, and the occasional Golgi complex. The characteristic paucity of organelles is a characteristic of most EC stem cells (de Chalain and Folb 1981).

5.62 THE ORIGIN OF PC13 EMBRYONAL CARCINOMA CELLS

PC13 is one of a number of clones isolated from a tumour OTT6050 by Stevens in 1957 and originated from the grafting of a six day old male embryo (strain 129/Sl C P) to a host (A/He x 129 - Sl C P) F_1 hybrid adult testis in 1967. The stem cells have since been maintained intraperitoneally in the 129/ Sv strain of mice by the serial transfer of ascitic fluid (Bernstine et al 1973).

Tumours of the OTT6050 A teratoma contain predominantly nervous tissue, although other types of differentiated cells have been observed. The OTT6050 B teratoma initially contained cells able to differentiate into many cell types, it was pluripotent and perhaps totipotent. The PC13 cell line is from this teratoma and these stem cells are thought to be mainly neuronal in nature (Stevens 1981, personal communication). This cell line was supplied to the University of Cape Town (Dept., of Pharmacology) in 1981 by Professor Fabian(University of the Witswatersrand).

5.71 THE DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS IN CULTURE

In culture it is possible to grow EC cells in relatively large numbers and this controlled environment allows the cells to be subjected readily to various biological and biochemical manipulations. It appears that cultured EC cells are highly stable with regard to the retention of developmental potentialities even after months of rapid proliferation in an undifferentiated state (Finch and Ephrussi 1967).

Occasionally , cell lines maintained in culture often undergo phenotypic and genotypic changes with the consequence that eventually the cells bear little resemblance to the starting population (Nicholas 1976). This phenomenom will be assessed for PC13 stem cells.

The discovery of physical and chemical means to induce specific differentiation in EC stem cell cultures and the characterization of the differentiated progeny has established the relevance of this system to normal development (Strickland 1981), and suggested that this in vitro model represents useful material for the study of teratogenic agents which affect differentiating cells. The process of cell determination, that is, the process by which a pluripotent cell becomes restricted in its possible determination may also be studied (Martin and Evans 1974).

5.72 DIFFERENTIATION INDUCTION

Aggregation was first used by Martin and Evans (1974) to induce differentiation of EC cells in culture. It is also possible to induce EC cells to differentiate by low density plating (Burk et al 1978, Pfeiffer et al 1981 , Speers 1979, and Ilgren and Littlefield 1981).

Hexamethylene bisacetamide treatment of an EC cell line resulted in the formation of differentiated cells with epithelial or fibroblast morphologies depending on whether or not the cells are aggregated during drug treatment (Speers et al 1979).

It was established in 1925 (Wolbach and Howe), that vitamin A compounds could influence differentiation and retinoic acid a vitamin A analogue induces differentiation of EC cells in culture (Strickland and Madhavi 1978). The effects of retinoic acid reported by Hogan et al (1981), are also dependent on cell aggregation. The effects of cell aggregation may result from inside - outside interactions similar to those hypothesized in other mammalian developmental processes (Herbert and Graham 1974).

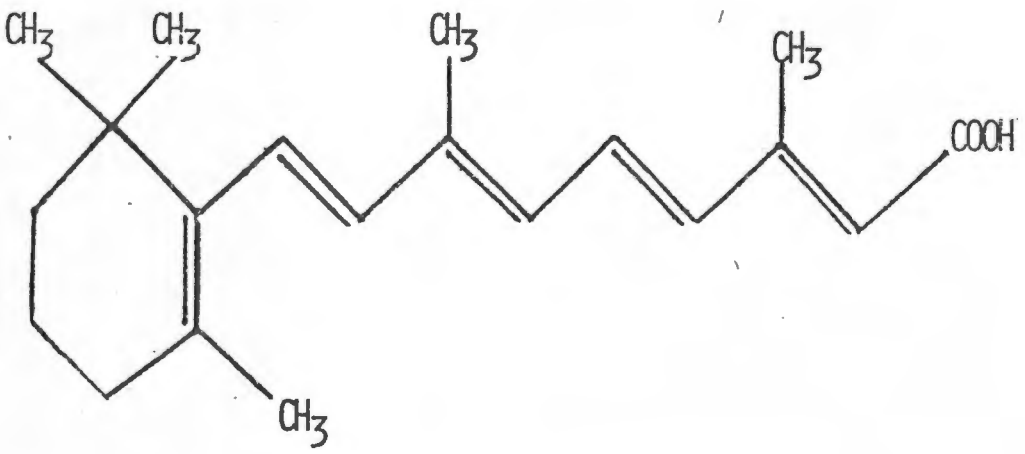
Efforts must be made to solve the problem of achieving unidirectional differentiation with cultures yielding largely or exclusively one cell type inorder that cytogenetic or biochemical analysis may be made on a differentiating homogeneous cell population (Mintz and Fleischman 1981). Conditions must be defined in which a certain type of differentiation occurs preferentially (Andrews et al 1982.).

The present study involved the differentiation of the PC13 EC cell line, a cloned cell line which can differentiate readily in vivo but requires the presence of retinoic acid for in vitro differentiation. The study by Strickland and Madhavi in 1978 showed that retinoic acid is increasingly effective as the concentration is raised to 10^{-6} M, above this amount it is toxic.

5.73 THE MECHANISM OF RETINOIC ACID INDUCTION OF CELL DIFFERENTIATION

The structural requirements for the effect of retinoic acid are stringent (Strickland and Mahdavi 1978). The carboxylic acid group is essential for activity as an inducer of differentiation. The structure can be modified in the cyclohexenyl ring system with retention of effectiveness.

Figure 8. THE STRUCTURE OF RETINOIC ACID



The intracellular consequences of retinoic acid treatment may be similar to the normal developmental signals which initiate differentiation (Jones-Villeneuve 1982). The process of normal cell differentiation involves selective transcription of different regions of the DNA to produce the messengers for the protein characteristics of the different cell types. Developmental processes require a succession of activity and inactivity of groups of genes. It is thought that binding proteins, similar to steroid hormones, may mediate the action of retinoic acid and that the effect is probably on the process of gene activation or inactivation which controls cell

differentiation by affecting cytoplasmic as well as cell surface components (Linder et al 1981). The biochemical mechanism of retinoic acid action however, is not yet understood (Strickland and Madhavi 1978).

The stimulus to differentiate appears to be indiscriminate: retinoic acid promotes whatever development the cells are capable of but does not influence the direction of differentiation (Mintz and Fleischman 1981). The time-course of the response by the cells shows that the amount of differentiation is proportional to the duration of treatment and suggests that retinoic acid facilitates formation of the differentiating cell type at each division. The continued presence of retinoic acid is not required for maintenance of the differentiated state and so is thought to act as a ' trigger ' (Strickland and Sawey 1980). The retinoic acid induced effects on F9 EC cell growth do not occur immediately but seem to manifest themselves afer a lag period of some twenty four hours(Linderetal 1981)

5.73.1 THE EFFECTS OF RETINOIC ACID ON CELL PROLIFERATION

Time - lapse cinematography has clearly shown that F9 EC cells with retinoic acid induced morphology continue to divide but that the rate of multiplication was considerably slower than that of untreated EC cells (Linder et al 1981). The number of cells grown in the presence of retinoic acid (10^{-6} to 10^{-8} M) has been shown to be similar to that of the control cells up to day two in culture. However, longer exposure to retinoic acid resulted in growth inhibition with increased time, thus, inhibition of cell growth occurred only after the cells were exposed to retinoic acid for more than two days (Ogiso et al 1982).

Differentiation of F9 EC cells induced by retinoic acid has caused a reduction in DNA synthesis levels as is evident from the measurement of the rate of ^{3}H-thymidine incorporation into cells. No decrease was observed during the first twenty four hours, but with futher culture, incorporation decreased. Futhermore, growth cultures revealed a slowing of the rate of cell proliferation (Linder et al 1981). The effects of retinoic acid on cell proliferation and rate of DNA synthesis would need to be established for PC13 EC cells used in this study and distinguished from those effects caused by exposure to DPH.

5.73.2 THE EFFECTS OF RETINOIC ACID ON THE SCE RATE

Retinoic acid has been found to neither induce an increase of SCEs nor cell cycle delay in Chinese hamster V79 cells with or without the metabolic activation using a S-9 mixture. However, it inhibited SCE increases and cell cycle delay in V79 cells induced by the indirect mutagen cyclophoshamide or aflatoxin B1. The inhibition was found to be dose and time dependent. These results suggest that retinol itself may have no direct effect on the genetic materials but rather exert its effects possibly by inhibiting the metabolic activation of an indirect mutagen or carcinogen (Huang et al 1982).

5. 74 INDUCED DIFFERENTIATION AND CELL TYPES

When retinoic acid was applied to cell lines with very little capacity to differentiate, for example F9, endoderm-like development occurred with associated biochemical changes (Strickland and Madhavi 1978), and this has been seen to occur in the PC13 EC cell line (Adamson et al 1979). This first cell type to differentiate from aggregated EC cells in culture resembles the endoderm cell layers which develop from the inner cell mass in the intact embryo and may contain a mixture of parietal and visceral endoderm-like cells (Adamson et al 1979). Since the normal embryonic equivalent of these cells is unknown, they are called END cells. They have also been referred to as ' giant ' because of their large size (Ilgren and Littlefield 1981, and Lo and Guila 1980). The term END or endoderm-like was used primarily for morphological distinction, but also based on biochemical criteria such as the production of plasminogen activator, a marker enzyme of parietal endoderm (Speers 1979). The END cells sometimes display " stress fibres " (Lo and Guila 1980) and numerous " thread-like extensions " (Linder et al 1981).

Retinoic acid at 10^{-6} M and low density plating (10^5 - 5 x 10^5 cells in a 25 cm^2 gelatin-coated tissue culture flask) caused large flat END cells to appear after just three days exposure. Binucleate endoderm-like ' giant ' cells are occasionally found in retinoic acid treated PC13 EC cell cultures (Ilgren and Littlefield 1981). ' Big flat cells ' were also observed with low cell density plating of EC cells by Papaioannou et al (1979).

Retinoic acid induced the differentiation of EC cells into a restricted spectrum of tissue types, namely, fibroblast-like cells, glial cells and neurones (Speers et al 1979, and Jones-Villeneuve 1982). Glial cells and neurone-like cells have been observed in retinoic acid treated cultures of the EC cell line P19, which does not differentiate into neurones in the absence of the drug. The morphology of these glial and neurone-like cells was also strikingly similar to the morphologies of cells in cultures of central nervous system and peripheral nervous system tissues (Jones-Villeneuve 1982).

It is well known that repeated transplantation can result in tumours that either do not differentiate at all and consists of EC cells (multipotential) or form predominantly neural tissue from EC cells and are called neuroteratomas (Stevens 1970, and Bernstine et al 1973), and this latter phenomenom may be the case for PC13 EC stem cells in culture upon stimulation of differentiation by retinoic acid.

5.75 BIOCHEMICAL MARKERS OF DIFFERENTIATION

A number of embryonic activities known to vary during embryonic development have been studied as biochemical markers. High alkaline phosphatase activity has been associated with EC cells but during differentiation it disappears very quickly and low levels of alkaline phosphatase have been shown to be properties of parietal endoderm. Other properties include the secretion of plasminogen activator and type IV collagen, and low levels of lactate dehydrogenase (Strickland et al 1980).

5.81 CYTOGENETIC CHARACTERISTICS OF EMBRYONAL CARCINOMA CELLS : UNDIFFERENTIATED CELL TYPE

There is evidence that teratocarcinomas at their inception tend to be chromosomally normal. It is particularly impressive that although chromosomal changes, including Y chromosome loss from X/Y lines may occur in these tumours, transplant lines have retained euploidy for very long periods (Mintz and Fleischman 1981). The basis for the exceptional karyotypic stability in teratocarcinoma cell in vivo remains unknown. It may be that in the teratocarcinoma transplant lines there is relatively little selective pressure favouring more rapidly proliferating variant cells (likely to be increasingly aneuploid) because a large percentage of the cells, when serially transferred in the host, are differentiating and are declining in mitotic activity during this limited period of residence of usually three to four weeks. The pool of dividing stem cells thus remains fairly small (Mintz and Fleischman 1981).

There is a large body of evidence indicating that chromosomal abnormalities in mammals result in failure or impairment of development. Therefore, in considering any teratocarcinoma cell line as a possible candidate as a differentiating " embryonic " model in teratogenicity testing and chromosome analysis, it is necessary to ascertain its karyotype before treatment application (Cronmiller and Mintz 1978).

Developmental pluripotency is accompanied by karyotypic normalcy or near-normalcy whilst aneuploidy has not yet given evidence of totipotency (Cronmiller and Mintz 1978). Karyotypic normalcy may therefore have predictive value for choosing teratocarcinoma lines with relatively high developmental prospects. However, slight deviations from euploidy need not impair the developmental potential (Cronmiller and Mintz 1978).

Guénet et al (1974) found normal or near normal karyotypes in their pluripotent EC cell lines but found many chromosomal anomalies in the F9 karyotype — these cells are no longer pluripotent. Although an abnormal karyotype is not inconsistent with the pluripotentiality of EC cells. The conclusion seems to be that EC cells with changed patterns of differentiation are always chromosomally abnormal, yet EC cells with abnormal karyotypes can remain pluripotent. It is certain that abnormal karyotypes by themselves do not prevent the expression of differentiated function since the EC cell karyotypes contain at least one copy of each pair of chromosomes and it is unlikely that the cells lack any gene that is present in the normal diploid cell. It seems probable that unbalanced karyotypes slightly alter the rate of various processes, and so change the apparent range of differentiation (Graham 1977).

The reason for the observed departure from karyotypic normalcy in EC cells in culture is probably due to the long-term culturing conditions where perhaps the nuclear material of the cell is suffering considerable disturbances which disrupt the mechanism of genetic control which may result in chromosome abnormalities (Guénet et al 1974).

Most EC cell lines are nearly diploid, having a modal chromosome number of 39 to 42, and a missing Y chromosome, whilst some carry one biarmed chromosome. This biarmed chromosome has been shown to occur with PC13 (Bernstein et al 1973), and may be termed a ' marker ' chromosome which describes any morphologically distinguishable abnormal chromosome that cannot be fully characterized. When markers in different cells are known or thought to be the same, they may be indicated by MAR, for example, MAR 1, MAR 2 etcetera (Schaeffer Hack and Lawce 1980).

An isochromosome is a chromosome inwhich the arms on either side of the centromere are morphologically identical by G-banding. Isochromosomes may be formed by two means; 1) by " centric fusion ", where two homologous chromosomes fracture at the centromere and the two similar short arms unite with each other and the other fragments are usually lost, and 2) a transverse fission through the centromere of a chromosome (perpendicular to the axis) in which the chromatids have formed. If the fractured chromatids unite with their adjacent (sister) strands, isochromosomes will result, where in subsequent metaphases the two joined chromatid arms act as a biarmed chromosome (Schaeffer Hack and Lawce 1980).

Isochromosomes of chromosome 8 have been detected in EC cells of the OTT6050 teratocarcinoma (Cronmiller and Mintz 1978). Two biarmed chromosomes, also termed the " metacentric " markers have been observed by McBurney and Adamson (1976). They also observed in the EC cell line C17S1 (of strain C3H/HE mouse) an elongated telocentric chromosome which could

be distinguished from the normal mouse chromosomes on the basis of length.

Many of the abnormal karyotypes of transplantable teratocarcinomas are trisomies (Iles and Evans 1977) which appear to be a heterogeneous collection, although trisomy 17 was common and a subline inwhich the EC cells formed mainly neural tissue had a modal chromosome number of 41, with trisomy for chromosome 11 (Graham 1977). Trisomy of chromosome 19 has been observed (Bernstine et al 1973) and of chromosome 8 (McBurney and Adamson 1976).

In teratocarcinoma cell lines, there has been found both additions to and deletions of chromosomes such as a 20% addition to the distal end of chromosome 1 and a 30% deletion from the distal end of chromosome 14 (thought at first to mimic a Y chromosome) (Graham 1977, and Papaioannou et al 1979). A deletion of chromosome 18 was observed by Guénet et al (1974) who also noted translocations of chromosomes 8, 11, 13 and 14. Intra-chromosomal rearrangement of chromosome 11 and others of the OC15 EC stemcell line of tumour OTT6050 was observed by McBurney and Adamson (1976), who also noted the addition of an extra band to chromosome 2, just below the centromere. A submetacentric marker chromosome has also been observed by McBurney (1977) in the C17S1 and C106S1 murine teratocarcinoma cell lines, thought to be formed by centromeric fusion of chromosomes 7 and 11.

PC13 EC stem cell cultures have also been reported to contain a small (>6%) subpopulation of polyploid cells (Ilgren and Littlefield 1981).

5.82 CYTOGENETIC CHARACTERISTICS OF EMBRYONAL CARCINOMA CELLS : DIFFERENTIATED CELL TYPE

Linder et al 1981 showed that retinoic acid induced differentiation in F9 EC cell cultures which was accompanied by a slowing down of the rate of cell multiplication. Strickland and Sawey (1980) concluded that the generated cell types of retinoic acid induced differentiation appear to be capable only of limited cell division in most cases. Ilgren and Littlefield (1981) suggested that the END or ' giant ' cells do not appear to be terminal cell types since they can occasionally enter renewed cell division inspite of their large size.

In some cases, so few of the differentiated in vitro progeny undergo mitosis, that it has been impossible to perform cytogenetic studies on them (Lehman 1974). However, it has been reported that the karyotypes of the differentiated derivatives of EC cells range from apparently normal to highly aneuploid (Lehman 1974, and Martin 1975). It is possible that the majority of the differentiated cells are arrested in the diploid stage and that the cells contributing to a proliferating tetraploid population are either specific cell types of differentiated cells or spontaneously transformed cells. It may be that an increase in chromosome number from diploid to tetraploid may be a necessary prerequisite for the differentiation of EC stem cells (Swartzendruber et al 1976).

Primitive endoderm has been found to become polyploid during the course of normal embryonic development (Ilgren and Littlefield 1981). Polyploid is the name for multiples of

the haploid number of chromosomes other than diploid, for example triploid, tetraploid, etc. Treatment of PC13 EC cell cultures with 10^{-6} M retinoic acid and low cell density plating (10^5 - 5 x 10^5 cells per 25 cm^2 flask) produced large END cells and polyploidation was about 16% of each cell culture. This net shift toward polyploidy resembled that which occurred during growth of visceral yolk sac endoderm by seventeen days post conception. As differentiated cells appeared in culture, cells in the near-tetraploid range appeared (Swartzendruber et al 1976). It should be noted that polyploidization involves an increase in DNA content of the cell, which has been found to correlate with an increase in cell size (Ilgren and Littlefield 1981).

5.9 SUMMARY

For obvious reasons it is not possible to use pregnant women and their conceptus for the direct screening of a drug and its teratogenic effect. Instead early monitoring systems have been used to study prenatal losses and are sensitive indicators of the response to a teratogenic agent. The monitoring of neonates with congenital abnormalities however has failed to detect minor changes in brain function or long-term carcinogenesis with only the major recognisable congenital malformations being recorded. Retrospective epidemiological studies on the malformed child have only been able to recognize easily observable abnormalities resulting in the identification of epidemics but not their causes (thalidomide an exception). With this type of retrospective monitoring the damage has already been done. Rather, prevention using teratogenicity

testing as the first step in defence followed by monitoring as the second step would be a much better approach to the prevention of teratogenic exposure.

In vivo animal systems have been developed which approximate the human situation. Here the variables are more easily controlled and the effects better indentified, however they have proved costly, time consuming and inefficient with no guarantee of protection from teratogenic exposure. In vitro animal systems (for example organ culture) have been devised which solve some of these problems but are not without their restrictions. Cell cultures have been proved of great value and can be used to monitor alterations in cell morphology, inhibition of the overall cell development and mutation induction. For the assessment of indirect teratogens with mammalian cell cultures, several experimental systems have been developed, such as liver microsomal and whole hepatocyte cell culture preparations. Differentiating cell cultures have been shown to have considerable potential in teratogenicity testing and may prove a very important adjunct to animal testing in the campaign against teratogenic agents.

It is thought that experimental results on teratogenicity in the mouse may have validity when extrapolated to man, and perhaps even more so than the costly and elaborate methods using subhuman primates.

Embryonal carcinoma (EC) cells have similar qualities to early embryonic cells in that they are pluripotent, structurally, biochemically and even immunochemically similar to some extent. A differentiating murine embryonal carcinoma cell culture would lend itself well to general microscopic morphological examination and cytogenetic analysis.

There are many physical and chemical means of inducing differentiation of EC cells and alterations in the characteristics of the differentiating progeny can be used to test for teratogenic agents. Retinoic acid as the inducer of differentiation may mimic the normal signals to differentiation which probably either activates or inactivates cell differentiation, however the precise biochemical mechanism is not yet understood. Retinoic acid promotes what development the cells are capable of and does not interfere with the direction of differentiation. The amount of differentiation is proportional to the duration of treatment, however the continued presence of retinoic acid is not necessary for the maintenance of the differentiated state. There is a lag period of about twenty four hours before differentiation is seen to occur after the "triggering" effect of retinoic acid administration. It should be noted that treatment with retinoic acid itself causes a slowing down and reduction of cell division after about fourty-eight hours and this would have to be distinguished from any similar toxic effect to the drug applied (in this instance DPH). In addition to this effect observed

at a morphological level, effects on DNA synthesis would have to be similarly evaluated. It should be noted that retinoic acid prevents SCE induction by the indirect mutagen cyclophosphamide upon chinese hamster cells in culture and could possibly have a similar effect on DPH mutagenic activity.

PC13 EC cells in culture are thought mainly to differentiate eventually into neuronal cells. They appear to be highly stable with regard to the retention of developmental potentialities after proliferation in the undifferentiated state. The first differentiating cell type appears after two to three days in cell culture and is endoderm-like resembling the four day inner-cell mass (endoderm) of normal embryonic development. These cells have been referred to as (Giant) because of their large size and possess "stress fibres" within their cytoplasm and "thread-like" cellular extensions. Fibrobast-like cells then make an appearance in differentiating cell cultures along with Glial-like cells and later Neuronal-like cell types. The morphologies of the latter cell types show a striking resemblence to cell cultures of tissues from the central and peripheral nervous systems.

Teratocarcinomas at their inception tend to be chromosomally normal and maintain exceptional karyotypic normality throughout their cell culture propogation. The more normal the karyotype, the less affected is the EC cell differentiation potential, however slight deviation from aneuploidy need not impair this.

Abnormal karyotypes have been shown not to prevent the expression of differentiative function but instead to alter the rate of the various processes and so limit the range of differentiation. EC cells may become karyotypically abnormal because the cells suffer from being in cell culture which disrupts the mechanism of genetic control resulting in chromosomal abnormalities. The fewer number of chromosomal anomalies found, the less would be this disruptive influence. This study examined PC13 EC cells in culture with regard to their normal (basal) level of chromosomal anomalies and any changes in frequency in response to the various treatments. In general, EC cells in culture show slight aneuploidy with biarmed chromosomes, elongated telocentric chromosomes, etc. and the chromosome count ranging from 39-42 per metaphase (normal mouse chromosome number per metaphase is 40).

In normal embryonic development, the primitive endoderm has been found to become polyploid, and the great increase in cell size (Giant) is due to the increased DNA content as a result of polyploidation.

Increased aneuploidy, structural aberrations and SCEs from the basal levels established for the PC13 murine EC cell line following particular cell culture treatments would be indicative of an (embryotoxic) effect and could possibly lead to the development of a valuable teratogenicity screening method.

CHAPTER 6.

EQUIPMENT/MATERIALS, METHODS AND EXPERIMENTAL PLAN

6.10 EQUIPMENT/MATERIALS

6.10.1 CELL CULTURING

Dry Incubator (set at 37°C and 60°C)	-	HERAEUS
Centrifuge	-	HERAEUS CHRIST GMBH
Vortex Mixer	-	ROTAMIXER DE LUX
Gas Bottle (5% CO_2 in air)	-	AFROX LTD,.
Air Filters	-	FLOW LABORATORIES.
Sartorius Balance	-	ZEISS
pH metre (model 601A)	-	ORION RESEARCH
Tissue culture flasks ($25mm^2$)	-	GREINER
Capped (sterile) conical plastic tubes (10ml)	-	GREINER
Plastic test tubes (5ml sterile)	-	GREINER
Glass Media Bottles (100 and 500 ml)	-	HOLPRO ANALYTICS(PTY)LTD,.
Plastic Surgical Syringes (sterile, 5,10 and 20 ml)	-	CPA HOSPITAL SERVICES.
Pasteur Pipettes (plugged)	-	HOLPRO ANALYTICS(PTY)LTD,.
Gelatine stock - powder working solution- 0.1% (w/v H_2O)	-	DIFCO
Hanks Balanced Salt solution (HBSS)(10-331-26)	-	FLOW LABORATORIES.
Trypsin/EDTA solution(1:10)	-	HOLPRO ANALYTICS(PTY)LTD,.
Dulbecco's Modification of Eagle's Medium(10-331-26)	-	FLOW LABORATORIES.
Eagle's Minimum Essential Medium(10-101-26)	-	FLOW LABORATORIES.

Table 10 . CONSTITUENTS OF EMEM AND DMEM CULTURE MEDIA (Flow Manual 1981).

INGREDIENT	EMEM * mg/L	DMEM mg/L
L-Arginine HCl	126.40	84.00
L-Cystine disodium salt	28.42	56.78
L-Glutamine	292.30	584.00
Glycine		30.00
L-Histidine HCl H_2O	41.90	42.00
L-Isoleucine	52.50	104.80
L-Leucine	52.50	104.80
L-Lycine HCl	73.06	146.20
L-Methionine	14.90	30.00
L-Phenylalanine	33.02	66.00
L-Serine		42.00
L-Threonine	47.64	95.20
L-Tryptophan	10.20	16.00
L-Tyrosine	36.22	72.00
L-Valine	49.90	93.60
D-Ca pantothenate	1.00	4.00
Choline chloride	1.00	4.00
Folic acid	1.00	4.00
Inositol	2.00	7.00
Nicotinamide	1.00	4.00
Pyridoxal HCl	1.00	4.00
Riboflavin	0.10	0.40
Thiamin HCl	1.00	4.00
$CaCl_2$ $2H_2O$	264.90	264.90
$Fe(NO_3)_3$ $9H_2O$		0.10
KCl	400.00	400.00
$MgSO_4$ $7H_2O$	200.00	200.00
NaCl	6800.00	6400.00
$NaHCO_3$	2000.00	3700.00
NaH_2PO_4 $2H_2O$	158.30	141.30
Glucose	1000.00	4500.00
Sodium phenol red	17.00	15.00
Sodium pyruvate		110.00

* with Earle's salts

Media Preparation

1. 900ml of deionized water was measured and the contents of the packet of powdered media (13.65g) were added (at room temperature) and mixed to dissolve.
2. 3.7g of Sodium Bicarbonate were added (as a buffer).
3. The pH was adjusted to 7.3 using either 1N HCl or 1 N Na OH.
4. The final volume of 1 litre was reached by adding more deionized water.
5. Using positive pressure to minimize the loss of CO_2 , the media was filtered through a sterile membrane filter (0.22 μm porosity).
6. Sterile powdered antibiotics were reconstituted with sterile distilled water and used at the following amounts in the prepared culture media.

   benzylpenicillin - 100 units per ml.
   streptomycin sulphate - 100 μg per ml.
   neomycin sulphate - 100 μg per ml.

   These antibiotics afford a good protection against a wide range of bacteria (gram positive and negative).
7. Sterile lyophilized Amphotericin B, reconstituted with sterile distilled water, was added to the culture media at 2.5 μg/ml.
8. Heat inactivated (56°C for 30 minutes to destroy complement activity) mycoplasma and virus free Foetal bovine serum (FBS) was added to constitute 10 % of the now ' complete ' culture media, providing essential growth factors necessary for cell culture .

Sodium Bicarbonate	-	MERCK
Benzylpenicillin(Sodium BP)	-	GLAXO
Streptomycin Sulphate	-	GLAXO
Neomycin Sulphate	-	LABETHICA
Fungizone(Amphotericin B)	-	SQUIBB
Fetal Bovine Serum (FBS)	-	U.C.T

6.10.2 FREEZING/THAWING CELLS

Freezing Ampoules (1ml)	-	GREINER
Dimethyl Sulphoxide(DMSO)	-	BDH

6.10.3 CELL COUNTING

Naeuber Haemocytometer	-	LEITZ DIAVERT
Coverglasses	-	HOLPRO ANALYTICS (PTY) LTD;.
Trypan Blue (vital stain) stock - powder working solution- 0.1% (0.1g in 10 ml HBSS)	-	MERCK

6.10.4 MORPHOLOGICAL, HISTOLOGICAL AND CYTOGENETIC OBSERVATIONS

Phase - contrast Microscope (with Photographic attachments)	-	LEITZ
Reticule Objective	-	LEITZ
'Ordinary' Light Microscope	-	LABORIX LUX 12 LEITZ

6.10.5 HISTOLOGICAL PREPARATIONS

Fixative (3:1) - methanol:acetic acid

Methanol - BDH

Glacial Acetic Acid - BDH

Giemsa stain - MERCK
stock - liquid
working solution- 10% v/v with PBS

6.11 DIFFERENTIATION INDUCTION

Retinoic Acid (R-2625) - SIGMA CHEMICAL COMPANY
stock - powder
working solution-10^{-3}M prepared in subdued light with absolute Ethanol, then diluted futher using medium with FBS.(Strickland and Sawey 1980).

6.12 DPH(PARENT COMPOUND) AND CHEMICALLY SYNTHESIZED METABOLITES.

Diphenylhydantoin(Dilantin) - PARK DAVIS COMPANY
or DPH.
stock - powder
Final dosage - 5,50 and 500 µg/ml of culture media.)

Solvents- (4:1:2:3) - BDH
Propylene glycol:
Ethanol:
NaOH (1N):
Water

Phenytoin, m- hydroxy metabolite* - WARNER-LAMBERT COMPANY

Phenytoin, p- hydroxy Metabolite* - WARNER-LAMBERT COMPANY

(* metabolites added to a Final dosage of 50 µg/ml)

EMIT®

This analysis involves a homogeneous immunoassay technique used inter alia for the microanalysis of DPH in biological fluids, but could also be used for culture media. A drug can be labelled with an enzyme then becomes bound to an antibody against the drug, with the activity of the enzyme becoming reduced. When the media is sampled, the drug present can compete with the enzyme-labelled drug for the antibody, thereby decrease the antibody-induced inactivation of the enzyme. The enzyme activity can be correlated with the concentration of the drug introduced and is measured by an absorbance change. Results were in µg/ml.

6.13 METABOLIC ACTIVATION

S-9 Mixture:-

S-9 Fraction	-	Dr. P. Thiel MRC - Institute for Nutritional Diseases, Tygerberg.
$MgCl_2$ (10.67mM)	-	MERCK
Phosphate buffer (133.33mM)- (titrated to ph 7.4 with 1N HCl)	-	MERCK
KCl (44.0nM)		
Disodium salt of NADP(5.33mM)	-	MANHEIM BOEHRINGER
Disodium salt of glucose-6 -phosphate(6.67mM)	-	MANHEIM BOEHRINGER
Ultracentrifuge	-	
Sterilization Filters	-	MILLIPORE

6.14 SOLVENTS

Ethanol (for Retinoic acid and drug preparation)	-	BDH
Propylene glycol (for drug preparation)	-	ALCHEMIST BDH
NaOH (for drug preparation)	-	MERCK

6.15 CONVENTIONAL CYTOGENETIC ANALYSIS

Colcemid Stock - 0.1% (10 µg/ml) Final amount added:- 0.06 µg/ml 0.1 µg/ml 0.6 µg/ml	-	CIBA- GIEGY
KCl (0.075M)	-	MERCK
Fixative (4:1) Methanol:acetic acid	-	refer to Section 6.10.5
Glass Slides (76mm x 25mm)	-	HOLPRO ANALYTICS(PTY)LTD
Coverslips (22mm x 22mm)	-	HOLPRO ANALYTICS(PTY)LTD
Mounting Media (DePEX)	-	HOLPRO ANALYTICS(PTY)LTD
Phosphate Buffered Saline(PBS) stock - tablet form working- 1 tablet to be made solution to 100 ml with H_2O	-	OXOID
Giemsa stain stock - liquid working solution- 10% v/v with pH 6.8 buffer	-	MERCK
pH 6.8 Buffer stock - tablet form working solution- 1 tablet made to 100 ml with H_2O.	-	HICKMAN & KLEBER LTD

6.16 G - BANDING

Trypsin: stock - 5% solution working solution 0.13% (1ml of stock/39 ml PBS)	-	BACTO-DIFCO

6.17 SISTER CHROMATID EXCHANGE

5- Bromodeoxyuridine (BrdU) 10 µM - SIGMA
(Shaeffer Hack and Lawce 1980)

Giemsa stain (GURR 66) - HICKMAN & KLEBER
stock - liquid
working solution- 2% v/v
diluted in:-
0.3M $Na_2 HPO_4$ - HICKMAN & KLEBER
(adjusted to pH 10.4 with
1N Na OH)

6.18 PHOTOGRAPHY

Enlarger - DURST with ILFO-SPEED ' Head '

Negative Holder - ROWI

Negative developer(G14 7C) - AGFA GEVAERT
stock - liquid
working solution- 75 ml of
stock and 225 ml H_2O

Negative Fixative (Hypam) - ILFORD
stock - liquid
working solution- 60 ml of
stock and 240 ml H_2O

Paper developer (Ilfospeed) - ILFORD
stock - liquid
working solution - 100 ml of
stock and 900 ml H_2O

Paper fixative (Hypam) - ILFORD
stock - liquid
working solution - 500 ml of
stock and 1500 ml H_2O

Print Paper (Glossy No.3) - ILFORD

Photographic Film
Pan F- Black and white - ILFORD
Colour - ILFORD

6.19 DNA SYNTHESIS

Spectrophotometer

Liquid Scintillation counter (model Ls 900 with automatic quench correction and DPM calculation facilities) - BECKMAN

^{3}H- Thymidine (specific activity = 5 Ci) - AMERSHAM, ENGLAND
stock - 250 µl with activity of 250 µCi (9.25 MEGAB Q) as an aqueous solution(sterile)
working solution- dilution of 1 in 10 to achieve an activity of 1 µCi per 10 µl.

Diluent: Saline (0.9g)(sterile) - PETERSEN LTD,

Millipore Media Filtering System - MILLIPORE
- Filters (0.02 µm porosity)

Filter Holder (XX 100 25 30)

Glass Fibre Discs (AP 150 22 00)

Flask with side arm (XX 102 50 5)

Trichloroacetic Acid (TCA) - MERCK PRODUCTS

Scintillation vials (20 ml) (low potassium glass) - BECKMAN

Scintillant- Demilum -30 liquid scintillation cocktail with chemiluminescence inhibitor - PACKARD INSTRUMENT COMPANY, INC

Sodium Dodecyl Sulphate (SDS) 0.5% solution with 0.1% DNA (to aid precipitation of cellular DNA.) - BDH

Serum Albumin(1μg/1μl SDS) - BDH

Solution X :-

A. 1% Copper sulphate - 1ml - MERCK

B. 2% Sodium/Potassium- 1ml - MERCK
Tartrate

C.-2% Na_2CO_3 - MERCK
100ml
-0.1 N Na OH

Mix A. and B. then
add to C.

Folin Ciscolteus Reagent - MERCK

stock - liquid
working solution- 1:2
of stock:water

6.2 METHODS

6.20.1 CELL CULTURING

1. To each of two new culture flasks were added 2.0 ml of a 0.1% gelatine solution. The flasks were immediately closed and carefully rotated in one plane inorder to spread the gelatine and produce an even and complete film over the bottom surface of the new flasks. Excess was removed after 5 minutes and discarded.

2. To each new flask was added 10 ml of fresh complete culture medium.

3. The old medium was removed from the confluent stock cell culture flasks and discarded.

4. 5 ml of HBSS were added to the stock cell culture flasks, the cap replaced and the solution gently swirled inorder to wash the cells and dilute the residual media protein that was present. The HBSS was then removed and discarded.

5. 2.5 ml of the trypsin/EDTA solution were added to the stock cell culture flasks which were then put into the 37 ^{o}C dry incubator.

6. After four minutes the flasks were removed and gently tapped to displace any cell patches adhered to the gelatinized surface.

7. The trypsin/EDTA solution was then diluted by the addition of 5 ml of fresh complete medium, inorder to reduce the enzymatic action of the trypsin.

8. The cell suspension was removed from the stock cell culture flask and put into 10 ml graduated capped conical plastic test tubes which were then centrifuged at 1500 rpm for 5 minutes.

9. The supernatant was poured off and the pellet of cells gently resuspended in 2 ml of fresh complete medium, afterwhich the tightly caped conical test tubes were vortexed inorder to thoroughly disperse the cells.

10. To each new gelatinized culture flask containing the 10 ml of fresh complete medium was added 1 ml of the well mixed cell suspension, and the flasks then ' gassed ' with an atmosphere of 5% CO_2 in air followed by incubation at 37°C in the dry incubator.

Note: In the controlled experiments, ' old ' flasks needing to be re-cultured were treated as 'stock cell culture flasks' above.

Sterile techniques were employed for all manipulations.

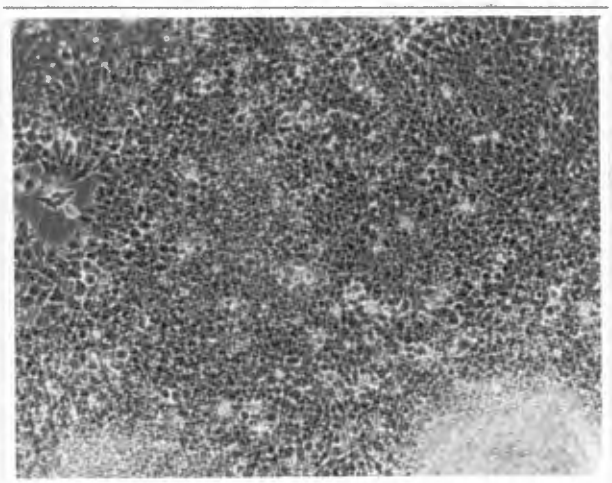
6.20.2 INCUBATION TEMPERATURE AND CELL GROWTH

The incubation temperature for cell culturing was very important since lower temperatures (such as 30°C) seemed to delay cell growth when compared with controls (at 37°C). Observations were made using the inverted phase contrast microscope and recorded in the following photographs (Figure 9). Temperatures above 42°C resulted in cell death with cells lifting off from the surface of the culture flask and floating freely in the media. It has been shown that cells grown at a temperature only slightly lower than optimum can decrease the growth rate and result in a lowered mitotic index and chromosomes with poor morphology (Shaeffer Hack and Lawce 1980).

Figure 9. INCUBATION TEMPERATURE AND CELL GROWTH

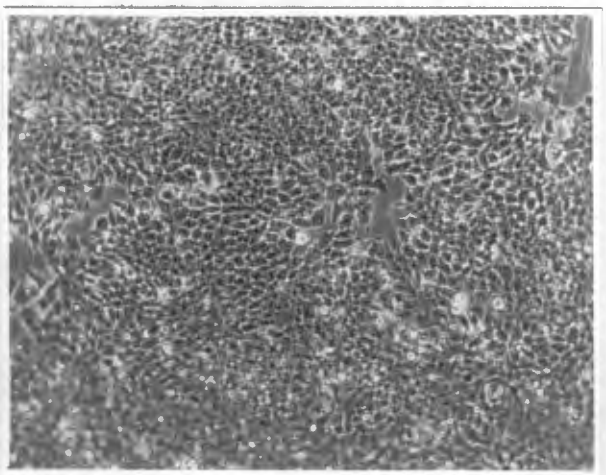
(initial cell density of 5 x 10^7 cells/ flask)

a. after 24 hours at 37°C



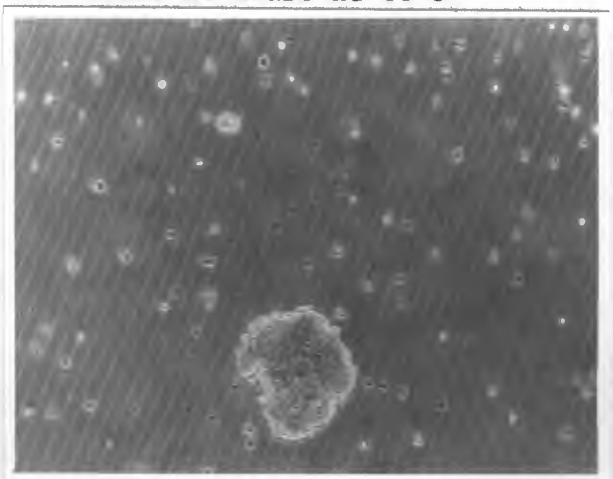
(x 80)

b. after 24 hours at 30°C



(x 80)

c. after 24 hours at 44°C



(x 80)

6.20.3 FREEZING CELLS

The PC13 cell line was preserved in this laboratory by the continuous freezing of stock cultures. In a frozen state they may keep for a long time without significant loss of viability (Flow Manual 1981.).

Freezing cells without cell death is possible due to the discovery that Dimethyl Sulfoxide (DMSO) protects cells from ice crystal formation during freezing. A concentration of 5% DMSO in fetal calf serum was used. Cells were kept at $^{-}90^{o}C$ to $^{-}196^{o}C$ (the temperature of liquid nitrogen) to prevent cell loss. Freezing units were used to cool cells at an optimal programmed rate, and special freezing ampoules were used which held about 1 ml of cell suspension each.

6. 20.4 THAWING CELLS

When required, cells were thawed by removal of the ampoule from the storage freezer and immediate immersion into a $37^{o}C$ waterbath, and agitated until defrosted. Cells were removed and added to a 5 ml tube with screw cap along with fresh complete medium, inorder to dilute the cryoprotective agent. Cells were then centrifuged, resuspended in 2 ml of fresh complete medium and plated into prepared culture flasks.

6. 20.5 CELL COUNTING

Cells were counted using a haemocytometer chamber which is divided into 9 large squares by triple white lines. The centre large square is divided into 25 small squares which, in their turn, are sub-divided into 16 smaller squares, whilst the four

corner large squares are divided into 16 squares. The large squares have each an area of 1 mm^2. The haemocytometer counting chamber was loaded as follows:-

1. The coverglass was placed down over the grid so that interference patterns appeared, giving the chamber a depth of 0.1 mm and each large square a volume of 10^{-4} ml.

2. A dilution was made of the cell suspension in vital stain (0.1 ml of cell suspension in 0.9 ml of Trypan blue solution), and a small drop added to the side of the coverglass. The chamber was filled by capillary action and care was taken not to overfill the chamber.

3. Under the low power objective of the light microscope, the cells were counted in the centre square and the four large corner squares. Cells touching the upper and right hand perimeter lines were ignored; those touching the lower and left hand perimeter lines were counted. Dead cells stained blue.

6.20.6 CALCULATION FOR THE NUMBER OF CELLS PER ML OF CELL SAMPLE

If the total number of cells counted was n and the solution was diluted y - fold in Trypan blue,
the number of cells in one large square = $\frac{n}{5}$,
the number of cells per ml of diluted suspension = $\frac{10^4 \, n}{5}$,
and the number of cells per ml of undiluted suspension = $\frac{10^4 \, n \, y}{5}$.

6.20.7 THE ESTIMATION OF PC13 EC CELL GROWTH RATE
(Method adapted from Priest 1977).

1. Cells were plated at an initial density of 10^6 cells per culture flask (in 8 replicate culture flasks), then gassed with 5% CO_2 in air and incubated at 37°C. This was taken as day 1, time 0 hours.

2. The cell count was performed on duplicate flask cell cultures at 24 hour intervals from the time of the initial plating for a total of 4 intervals (the entire analysis taking 5 days).

3. The average of the duplicate cell count for each 24 hours was plotted (on the vertical log scale) against time in culture in hours (horizonatal scale). Including the initial number of cells at 0 time, 5 intervals were plotted on the semi-log paper (Results, page 175).

6.20.8 MORPHOLOGICAL OBSERVATIONS

Culture development was monitored on day 12 of Experiments A, B, C, and D. The following parameters were measured:-

i. CELL DEATH — This was confirmed by the observation of floating cells in the medium which had lifted off from the flask surface and appeared as small round refractile bodies. Quantitatively, a broad description of cell death was employed which with cell count determinations was used to estimate cell culture growth.

ii. GROWTH RATES — Cell culture growth rates were compared for each treatment by an estimation of cell density for which cell counts were performed in Experiments A and B, and protein determinations made for Experiments D and E (correlating with cell density). Both measurements were an indication of the rates of cell proliferation.

iii. CELL MORPHOLOGY- The general appearance of cell cultures was noted and a comparison made with controls. Normal EC cell morphology (illustrated by the controls) was distinguished from differentiating cells and a quantitative estimation of each type present was made using the technique described in Section 6.21.2.

6.20.9 HISTOLOGICAL PREPARATIONS

1. Media was removed from the culture flasks and the bottom layer of the flask was quickly cut with a hot scalpel blade into approximately 20mm x 40 mm sized pieces.
2. These preparations were then fixed with Methanol:acetic acid (3:1) in a glass petri dish for 30 minutes.
3. Fixative was removed and Giemsa stain added for 4 minutes.
4. The stain was poured off and a water rinse followed.
5. The preparations were left to air dry.

6.21 DIFFERENTIATION INDUCTION

Retinoic acid was used to induce in vitro differentiation of PC13 stem cells. In experiments A, B, C and D, retinoic acid was added on Day 1 and DPH or its metabolites were added on Day 7. In some treatments, retinoic acid was added for a futher five days (until Day 12) afterwhich the cell cultures were terminated and the analyses performed (see Table 13.).

6.21.1 RETINOIC ACID CONCENTRATION

The concentration of retinoic acid used in this laboratory to induce PC13 EC cell differentiation prior to this investigation was 1.33×10^{-6} M. It was decided upon to test three retinoic acid concentrations, viz 10^{-5} M , 10^{-6} M , and 10^{-7} M. The 10^{-6} M concentration was that used by Strickland and Mahdavi (1978) for inducing the differentiation of F9 EC cells. The higher concentration was used to test for toxicity and effect on differentiation, whilst the 10^{-7} M concentration was used to examine the potential of retinoic acid to induce differentiation at low concentrations.

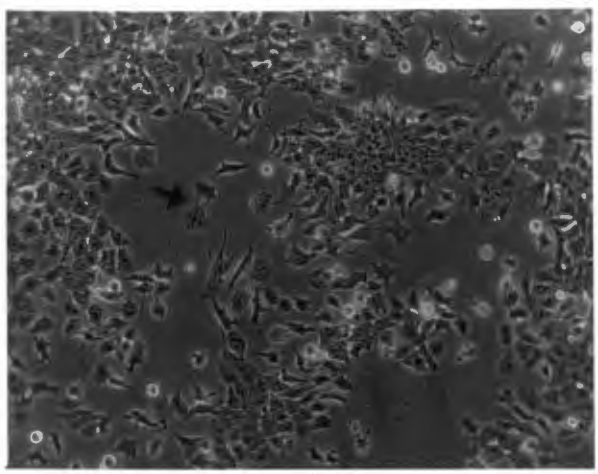
Chronic exposure to retinoic acid was employed as this would ensure adequate inducement of differentiation in the relatively short period allowed for an experimental run.

Direct observation of growing cell cultures (Day 12 of cell culture) using the phase contrast microscope revealed that with a retinoic acid concentration of 10^{-6}M there was a large number of easily visible differentiating cells after 12 days of mainly fibroblast-like character (Section 6.21.2) surrounding

clumps of undifferentiated EC cells (junction between two cell types indicated by the arrow in Figure 10.b.). Lower retinoic acid concentration (10^{-7}M) resulted in much less differentiation of an earlier stage, indicated by the presence

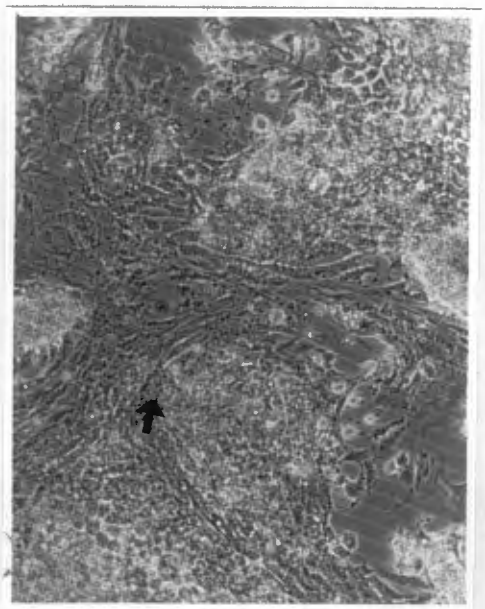
Figure 10. RETINOIC ACID CONCENTRATION : CELL GROWTH AND DEVELOPMENT
(initial cell density of 5×10^6 cells/flask)

a. 10^{-7}M retinoic : Day 12



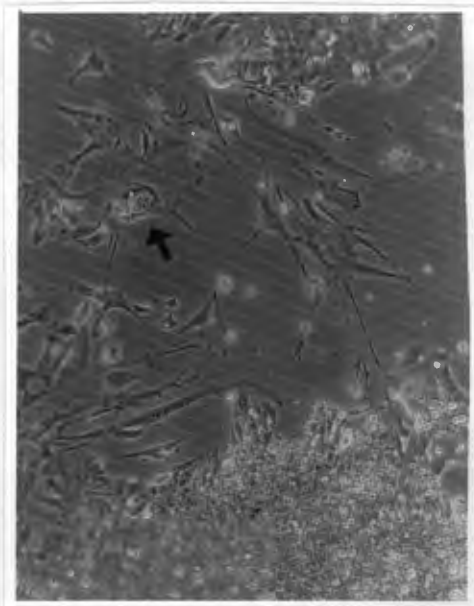
(x 80)

b. 10^{-6}M retinoic acid : Day 12



(x 80)

c. 10^{-5}M retinoic acid : Day 12



(x 80)

of small granular Endoderm-like cells (indicated by the arrow in Figure 10 a.). The relatively low level of differentiation in this instance was inappropriate for the time scale planned for experimental runs. The highest retinoic acid concentration employed (10^{-5}M) resulted in rapid differentiation of cells many ofwhich were in the large Endoderm or END - like form.(indicated by the arrow in Figure 10 c.) surrounding large clusters of undifferentiated EC cells many of which in the top layers had lifted off due to cell death and were floating around as small refractile bodies in the media. A retinoic acid concentration of 10^{-6}M was employed for further experimental work.

6.21.2 ANALYSIS OF DIFFERENTIATION

Differentiation of EC cell cultures was qualitatively analysed by the direct observation of morphological changes in the cells following differentiation induction. The descriptions and nomenclature employed here closely follows that used by the authors referred to in Section 5.74. The following cell types were observed :-

Undifferentiated EC cells (10-12 μm)

Small granular Endoderm-like cells (20-30 μm)

Glial-like cells (30-60 μm)

Fibroblast-like cells (50-200 μm)

Large Endoderm-like cells (100-200 μm), with " Stress fibres " and " Thread-like extentions ".

Large multi-processed Neuronal-like cells (100-200 μm)

Differentiation of monolayer cultures of cells was quantified by a point counting method (Hennig et al 1968, and Speers et al 1979). Growing cultures in tissue culture flasks were examined using an inverted phase contrast microscope with a reticule grid in one ocular lens. The flask was positioned at random and each of 121 points of intersection was scored for the underlying cell type. The frequency of " hits " was directly proportional to the area covered by each cell type. The relative areas covered by EC (undifferentiated embryonal cells) and DIFF cells (differentiating embryonal carcinoma cells), was estimated by photographing confluent areas of each at the same magnification and counting the nuclei in these pictures. The average for several determinations was 3.5 EC cells per Diff cell.

The relative proportion of EC cells in a mixed EC/DIFF cell culture was computed using the following formula:-

$$\frac{\text{EC hits} \times 3.5 \times 100}{\text{EC hits} \times 3.5 + (\text{DIFF hits})} = \% \text{ DIFF cells}$$

$$= \% \text{ Differentiation}$$

6.22.1 DPH (PARENT COMPOUND)

DPH, thought to be responsible for the set of congenital abnormalities commonly referred to as the Fetal Hydantoin Syndrome, was here tested for toxic effects.

DPH was initially added to cell cultures at a concentration of 5, 50 and 500 μg/ml and its effects on chromosome aberration induction, DNA synthesis and general morphological appearance determined. These concentrations were chosen because 50 μg/ml of DPH was used by Wilk et al (1980) when examining the effects of DPH on chick mesenchymal and neural crest cells in culture with regard to changes in cell morphology, differentiation inhibition and cell death. DPH at a concentration of 50 μg/ml caused effects on these parameters, and so it was decided upon to use this concentration as the intermediate ' dose ' of DPH used in this study. This concentration of DPH was in the range of blood levels that have been associated with congenital malformations (Wilk et al 1980). The two other concentrations of 5 and 500 μg/ml were included in the present study to give an indication of a dose response relationship to DPH exposure.

The development of the cells was assessed by phase contrast microscopy for 5 days after the addition of DPH to cell cultures (after Wilk et al 1980) and represented chronic exposure in relation to the observed rate of cell differentiation.

6.22.2 CHEMICALLY SYNTHESIZED METABOLITES OF DPH

Two of the main DPH metabolites in man, p- HPPH and m- HPPH, were available chemically in small quantities and were included in Experiment D. as positive controls for the metabolic activation treatment involving the S-9 Mixture. A concentration of 50 μg/ml was used and the development of cells assessed by phase contrast microscopy for 5 days after the addition of the metabolites.

6.23 METABOLIC ACTIVATION OF DPH

Metabolic activation of DPH using liver microsomal preparations should result in the production of intermediates of metabolism and various end metabolites. These metabolites were not identified qualitatively or quantitatively, however, it was possible to measure quantitatively the disappearance of DPH using EMIT (refer to Section 6.12.1).

The metabolism of DPH by the S-9 Mixture was monitored, as a function of time by measuring the decrease in DPH concentration at hourly intervals in the sample having an initial DPH concentration of approximately 52 μg/ml. It was found (Table 11; Figure 11.) that the drug concentration diminished to about only 10% of its original value after 2 hours of incubation with the S-9 Mixture. It was therefore assumed that metabolic activation had indeed occurred and that metabolites were present in the highly centrifuged (50,000 g) supernatant of the S-9 Mixture with the drug added.
As discussed in Section 2.5 an end metabolite of DPH metabolism

may be able to revert back to the reactive arene oxide intermediate which may be the teratogenic component. All futher work with the S-9 Mixture (Experiments A., B., C., and D.) involved the incubation of the S-9 Mixture with DPH for 4 hours prior to centrifugation, to ensure that all activity had stopped due to lack of enzyme or substrate and that the DPH added would have been reduced to a probable 10% of the original amount present.

Table 11. DPH AND METABOLIC ACTIVATION WITH S-9 MIXTURE

TIME (hours)	DPH (μg/ml) (1)	(2)	AVERAGE
0 (no S-9 Mix)	50.4	53.4	51.9
0 (with S-9)	38.4	40.2	39.3
0.25	24.4	23.3	23.9
0.5	15.5	16.1	15.8
1.0	10.8	10.9	10.9
2.0	6.6	6.2	6.4
3.0	7.3	9.3	8.3
4.0	8.1	7.0	7.6

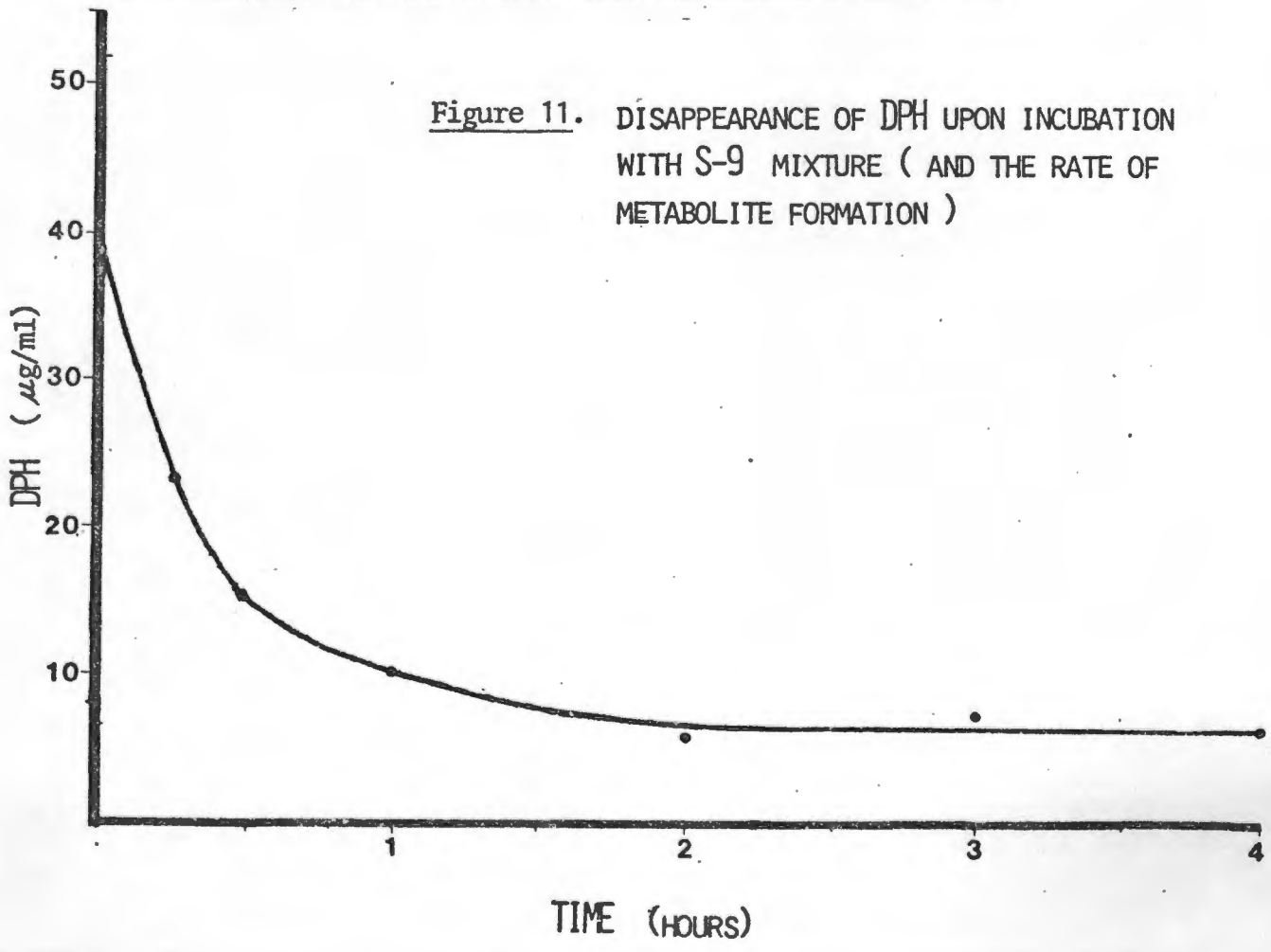


Figure 11. DISAPPEARANCE OF DPH UPON INCUBATION WITH S-9 MIXTURE (AND THE RATE OF METABOLITE FORMATION)

6.23.1 PREPARATION OF RAT LIVER MICROSOMAL FRACTIONS

The S-9 fraction was kindly supplied by Dr. P. Thiel,
Medical Research Council,
Institute for Nutritional Diseases,
Tygerberg, CAPE.

The liver microsomes were obtained principally after the method developed by Ames et al (1975), as follows:-

1. Ten BD 1X male rats of about 200g body weight each were fed on rat cubes and five days before they were killed, 0.1% sodium phenobarbital was added to their drinking water in order to cause liver enzyme induction. On the fifth day the rats were killed and the livers excised aseptically, and processed at 0 - 4°C.

2. The livers, with an approximate weight of 10 - 15 g each were divided into two groups of five each and transferred to pre-weighed 250 ml glass beakers containing 0.15 M KCL (1 ml/g wet liver).

3. The weighed livers were then transferred to glass beakers containing 0.15 M KCL (3 ml/g wet liver), and blended in a Sorvall - Omni - mixer for 30 seconds at full speed.

4. The mixture was filtered through cheese cloth into a 250 ml glass beaker and homogenized in a Daunce homogenizer using 10 strokes with a tight plunger.

5. The homogenate was centrifuged in a Sorvall SS-34 rotor for 10 minutes at 9000 g and the supernatant (S-9 fraction) was transferred in 3 ml quantities into test tubes with metal caps and stored at -80°C until required.

6.23.2 PREPARATION OF THE S-9 MIXTURE

The rat liver microsomal samples (S-9 fractions) were supplemented with certain cofactors and salts forming the S-9 mixture reqired for the metabolic activation of DPH. The S-9 mixture was prepared at $4^{o}C$ and contained (per 1 ml of complete media) the following:-

0.2 ml of S-9 fraction, and

0.025 ml of 10.7 mM $MgCl_2$,

133.0 mM phosphate buffer,

44.0 mM KCl,

5.3 mM disodium salt of NADP and

6.7 mM disodium salt of glucose-6-phosphate.

The volume of S-9 fraction, salt solution and cofactors used per ml of culture media was the same as that used by Wilk et al (1980), whilst the addition of KCl and phosphate buffer and the molarity of all components used follows the method of Ames et al (1975). Due to the instability of NADP when in solution, the S-9 fraction was prepared on the day of use and the components (other than the S-9 fraction) were made up in 10 ml of distilled water.

S-9 fraction added directly to the medium is toxic to cell cultures and so the S-9 mixture was initially incubated with the drug to be tested (DPH) or vehicle control (5 μl per 0.225 ml of S-9 mix) and then highly centrifuged (50,000 g or 27,000 rpm) for 1 hour . 0.23 ml of the supernatant was added per ml of medium which was then sterilized by filtration. Prepared in this way the S-9 fraction would not be toxic to cells in culture (Madle and Obe 1977).

6.24 SOLVENTS

Solvents used in this investigation included ethanol, propylene glycol and sodium hydroxide. Equal amounts of the various solvents were tested in all experimental runs inorder to examine any differences from the negative controls. If the solvents had no effect then their usage would only be limited by toxicity at high levels (Latt et al 1981).

All three of the above mentioned solvents, used at similar concentrations to those used in this investigation, had no effect on developing cells (chick mesenchymal and neural crest cells) according to Wilk et al (1980).

6.25 CONVENTIONAL CYTOGENETIC ANALYSIS

Although there are many published methods for cytogenetic analysis of teratocarcinoma cells (Evans 1972; Lehman et al 1974; Papaioannou et al 1979; Cronmiller and Mintz 1978, and McBurney and Strutt 1980), these techniques are only glossed over in this literature. Detailed knowledge of these techniques is however critical for obtaining good metaphase preparations. Various differences in experimental conditions exist (such as different brands of reagents employed , different environmental climates etc,.), therefore investigators need to establish specific cytogenetic procedures for their laboratory conditions and for the actual cell line used.

The treatments involved in the harvesting procedure, although mostly routine cytogenetic techniques, had to be specially adapted for the murine teratocarcinoma cell line used in this study. Harvesting of the cells according to the exact methods of the Human Cytogenetics laboratory at the University of Cape Town Medical School did not afford good chromosome preparations, due probably to the inherent characteristics of the cell line used.

It was necessary to establish each stage of the Harvesting procedure, such as colcemid concentration and exposure time, KCl exposure time, and fixative exposure time, before proceeding to the main experimental work with specific drug treatments. Eventually, a harvesting and slide making procedure was established which gave relatively good and consistent results.

6.25.1 COLCEMID — EXPOSURE TIME AND CONCENTRATION

The first step in the harvesting procedure is the addition of the mitotic inhibitor which accumulates metaphases and prevents the formation of the spindle apparatus by blocking assembly of microtubule monomer units into polymeric spindle fibres.

Colcemid was added 0.06, 0.1 and 0.6 μg/ml to culture flasks after the cells were plated at 10^6 cells/flask. Cultures were incubated for $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3, and 4 hours, then harvested. Observation of conventionally stained slides under oil-immersion lens of the light microscope showed that better and adequate numbers of metaphase plates occurred at 0.1 μg/ml colcemid concentration and exposure time of 3 hours. Many of the chromosomes appeared well spread out and at the length which allowed adequate examination of their morphology. Before this time and with lower colcemid concentration metaphase plates were fewer in number and the chromosomes were long and not well spread out, with a residual cytoplasmic background. Increasing exposure time beyond 3 hours and increasing colcemid concentration seemed to cause most of the many metaphase plates observed to be too compact, and chromosomes too short.

6.25.2 KCl (0.075 M) EXPOSURE TIME

During mitosis, one of the changes that takes place in the cell is the disintegration of the nuclear membrane. The chromosomes are then held together by the cytoplasmic membrane and the spindle apparatus. The addition of this hypotonic solution causes water to enter the cell and the cytoplasmic membrane stretches and the cell swells.

Six exposure times (5, 10, 20, 30, and 60 minutes) were tested using pre-warmed KCl (0.075 M) and incubation in the dry incubator at 37°C. Centrifugation time of 5 - 7 minutes was included as part of the exposure times. It appeared that a total exposure time of 20 minutes (which later also included an initial rinse in KCl to dilute any media still present), proved to have the best effect, producing many well spread but apparently complete metaphase plates. Times less than 20 minutes resulted in underspread metaphase plates and crossed chromosomes with visible cytoplasm. Beyond this time many of the metaphase plates appeared incomplete with many scattered chromosomes present and poor chromosome morphology.

6.25.3 FIXATIVE

Prior to fixation, cells are alive, metabolizing and continue to be affected by the mitotic arresting agent. The fixative preserves the swollen cells, stopping all cell functions, preventing futher cell swelling and making the cells less fragile (although the cells must still be treated gently as they are sensitive to the first fixation).

The addition of a few drops of fixative at the end of KCl exposure seemed to help the cells centrifuge out of hypotonic suspension and make them less fragile during the fixation steps (Shaeffer Hack and Lawce 1980). It appeared that the more times the cells were fixed, the more distinct and better spread out the chromosomes looked after staining, although, if left in fixative for too long before spreading, the chromosomes appeared fuzzy. Insufficient fixation resulted in many crossed chromosomes,

visible cytoplasm, holes in the chromosomes and poor morphology.

An increase in methanol concentration to achieve a ratio of 4 : 1 with acetic acid was found to help prevent overspreading and allowed better clearing of cellular residues, giving ' cleaner ' metaphase preparations than with the 3 : 1 ratio usually employed.

Cell storage : in fixative overnight (for convenience) as a pellet instead of a loose suspension, was thought to protect cells from overfixation and so helped to keep them from becoming trypsin-resistant and perhaps aided SCE differential staining (Shaeffer Hack and Lawce 1980).

6.254 THE HARVESTING PROCEDURE

1. At the appropriate time, 0.1 µg/ml Colcemid was added to the cell cultures which were then gently shaken and reincubated for a futher 35 minutes at 37°C.

2. Instructions 3. to 7. of the method for cell culturing (section 6.201) were performed with the exception that the old media or HBSS were not discarded but put into the centrifuge tubes and centrifuged at 2,000 rpm for 7 minutes. The supernatant was then removed down to the 1 ml mark and discarded.

3. The cell suspension was removed from the culture flasks and put into the centrifuge tubes already containing the 1 ml old media/HBSS rinse cell solution.

4. The remainder of the procedure did not require aseptic conditions, continuing with centrifugation of the cell suspension at 2,000 rpm for 7 minutes. The supernatant was carefully pipetted off down to the 0.1 - 0.5 ml mark depending upon the size of the cell pellet.

5. The cells were gently but thoroughly resuspended with the addition of 0.5 - 1.0 ml of warmed KCl (0.075 M). Warmed KCl was then added up to the 8 ml mark, whilst working as rapidly as possible with the pasteur pipette inorder to thoroughly mix the solution.

6. The tubes were vortexed for 10 seconds and then immediately centrifuged at 2,000 rpm for 7 minutes.

7. All but 0.5 - 1.0 ml of the supernatant was carefully removed from the tubes and the cells gently resuspended. Warmed KCl (0.075 M) was added, mixing constantly, up to the 8 ml mark (less if the cell density was low).

8. Tubes were vortexed for 10 seconds and put into the 37°C dry incubator for 6 minutes. After 3 minutes, the cells were agitated by tapping inorder to prevent the cells from settling out and escaping from the KCl (0.075 M) concentration gradient.

9. The cells were removed from the incubator and a few drops of fresh cold fixative were added to each tube which was then centrifuged at 2,000 rpm for 5 minutes.

10. ' First Fixation ' - the critical step in the procedure. The supernatant was carefully pipetted off to the 0.5 - 1.0 ml mark, depending upon the cell pellet size, and the cells gently mixed. More cold fixative was vigorously added to the 8 ml mark and mixed well.

11. Tubes were vortexed for 10 seconds and left at room temperature for 15 minutes. The cell suspension was then centrifuged at 2,000 rpm for 7 minutes.

12. ' Second Fixation '

Instructions 10. and 11. were repeated.

13. The cells were refrigerated (about 4°C) as a pellet for 24 hours, then the following day the settled cell suspension was centrifuged at 2,000 rpm for 7 minutes.

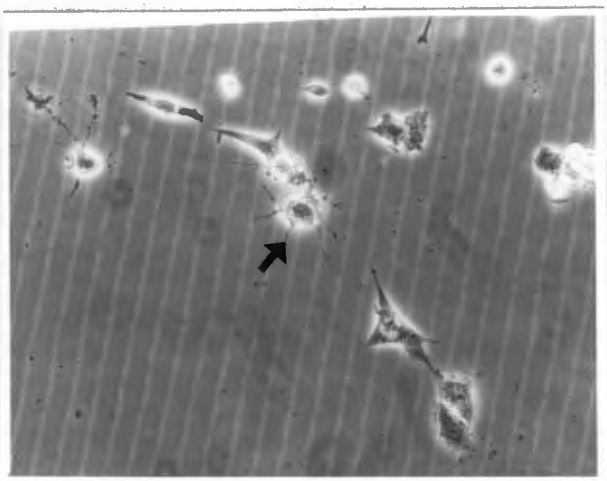
14. The supernatant was removed down to the 0.5 - 1.0 ml mark and the cells gently resuspended in this volume, which when necessary was adjusted inorder to obtain a slightly opaque solution. This was followed by slide ' spreading '.

6.25.5 MITOTIC ' SHAKE OFF '

In an attempt to increase the number of metaphase plates for cytogenetic examination, mitotic ' shake off ' was employed to synchronize cell division by replating the mitotic cells which were detached from the culture layer by tapping the flask, since the cells in metaphase had been loosely attached to the growth surface. However, this led to cell differentiation (Figure 12.a. b. and c. where arrows indicate a differentiating cell.), probably due to the ' low cell density effect ' (Section 5.72). This method did not prove to be a way of increasing the mitotic index, and it helped to confirm the phenomenom of differentiation induction due to low cell density plating.

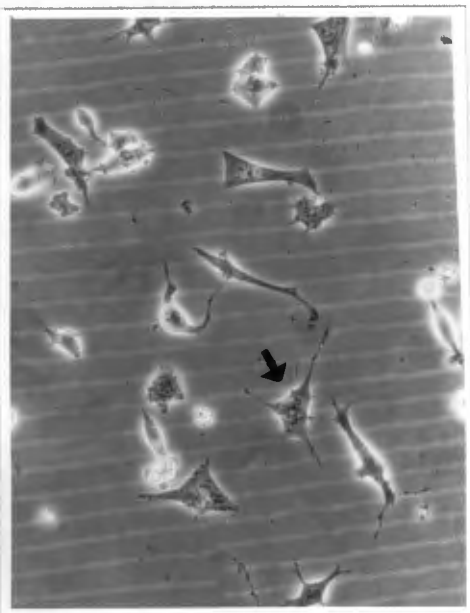
Figure 12. MITOTIC ' SHAKE OFF '

a. 3 hours after plating



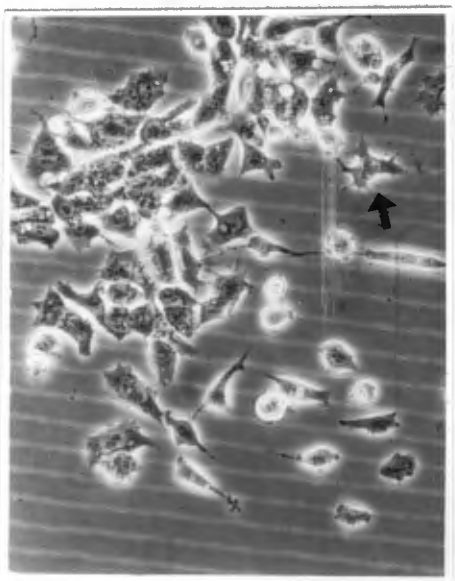
(x 250)

b. 8 hours after plating



(x 250)

c. 24 hours after plating



(x 250)

6.26 SLIDE SPREADING (CONVENTIONAL ANALYSIS, G- BANDING AND SCE)

The method of air - drying chromosomes spread on pre-cleaned glass slides allowed the fixative to be lost quickly due to its property of quick evaporation from around and under the swollen cells which then flatten completely, forcing the chromosomes to spread out. This occurred after the cells were dropped onto the cold slides by the " bonsai " technique which involved dropping the cell suspension from a height. Different methods of making slides and the environmental climate may speed up or slow down the process of evaporation, causing a greater or lesser spreading effect (Shaeffer Hack and Lawce 1980).

Neither fast nor slow evaporation is ideal, since either can result in poor spreading , scattered chromosomes, or visible cytoplasmic background around the metaphase.

In this study, the method involved dropping the cell suspension onto cold wet slides lying on a wire rack over a bowl of hot water to allow the slides to dry more slowly in the rather hot climate. When the slides were dried incorrectly, the chromosomes tended to look indistinct or fuzzy.

One slide was prepared initially and examined for cell density and if the quantity and quality of mitotic plates were satisfactory, all of the cell suspension was spread onto slides. The quality of the metaphase plates could sometimes be improved by re-fixation or by using fixative composed of Methanol and

Acetic acid in a 1 : 1 ratio instead of the usual 4 : 1 ratio, this would tend to prevent underspreading. Slides were stored in a 60°C dry incubator until ready for use, this was thought to ' age ' the slides and afford better staining.

Chromosome spreading and morphology,and therefore banding quality are completely dependent upon how slides are made and a very important end point in cytogenetic analysis is reproducibility inorder to obtain consistent results, however this is quite difficult to achieve.

6.27.1 SLIDE STAINING : CONVENTICNAL ANALYSIS

1. The slides were put into 10 % Giemsa stain in a pH 6.8 buffer for 4 minutes.

2. Slides were then removed and rinsed under running tap water.

3. Tissue paper was then used to blot the slides which were left to dry at room temperature.

4. Slides were mounted with a coverglass using mounting media.

6.27.2 SLIDE SCANNING : CONVENTIONAL ANALYSIS

A uniform method was followed when scanning with a low power objective on the light microscope, to ensure that the slide was totally examined. All results were recorded on a tally sheet (see Appendix). Slides were analysed ' blind ' and all metaphases, ofwhich chromosome morphology could be definitely distinguished were analysed. Photographs were taken where necessary using the x 100 oil immersion objective.

6.27.3 G- BANDING

G - banding results from the digestion of chromosomes with a proteolytic enzyme (Trypsin) and then staining with Giemsa stain. This technique reveals detailed information, the banding pattern, which could afford identification and classification of the karyotype of the cells studied. The optimal resolution of bands is obtained from early metaphase chromosomes which are longer. In contracted chromosomes, the finer bands tend to merge together so that only the main bands are apparent.

1. It was necessary to 'age' the slides in a dry oven at 60°C for 24 hours inorder to eliminate fuzzy banding and to enhance the contrast of the bands.

2. The slides were then dipped into the working solution of freshly prepared Trypsin (0.13%). Timing was critical, about 30 seconds for the preparation recorded in this study. Overtrypsinized chromosomes appeared bloated and undertrypsinized chromosomes stained uniformly with little evidence of banding.

3. Slides were then rinsed thoroughly in a PBS rinse.

4. Slides were stained in 10% Giemsa stain, diluted in pH 6.8 buffer, for 3 minutes by flooding the horizontal slide.

5. Slides were then rinsed in gently running tap water and blotted dry, and lastly, mounted in the usual way.

6.28.1 SISTER CHROMATID EXCHANGE

Detection of SCEs in non-ring chromosomes requires some means of differentially labelling sister chromatids. This was initially accomplished with the use of tritiated thymidine (Taylor et al 1957), although recent studies employ halogenated nucleosides such as 5-bromo-2'-deoxyuridine (BrdU). BrdU has largely supplanted ^{3}H- thymidine for the purpose of differentially labelling of sister chromatids since when incorporated into DNA the BrdU can quench the fluorescence of DNA binding dyes such as 33258 Hoechst (Latt 1973), acridine orange (Dutrillaux et al 1973; Kato 1974b, and Perry and Wolff 1974), and under appropriate conditions 4'6- diamidino-2- phenylindole (Lin and Alfi 1976). Fluorescence methods for SCE detection do not provide permanent preparations and rapid fading of stained specimens makes even initial photomicroscopy difficult. Giemsa methods (Goto et al 1978; Ikushima and Wolff 1974; Korenberg and Freedlender 1974; Perry and Wolff 1974; Sakanishi and Takayama 1978, and Scheres et al 1977), many utilizing a BrdU - sensitive prestain for photosensitization, have thus in many laboratories replaced fluorescence techniques for routine SCE analysis.

In this study, a direct technique was used for the selective staining of BrdU - substituted DNA and involved treatment with salts at a high pH to enhance the staining when Giemsa stain alone was employed (Alves and Jonasson 1978).

1. Brdu was added (10μM) to cell cultures 40 hours before harvest. Thereafter, the cultures were kept in complete darkness.

2. After harvesting by the usual method, the slides were stained 45 minutes at room temperature.

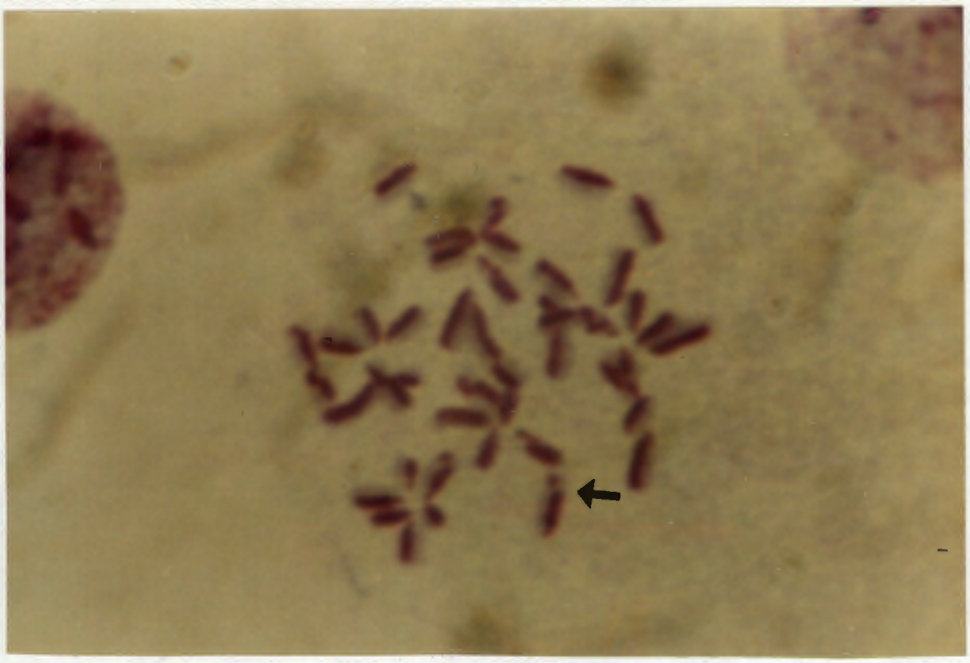
3. The preparations were then rinsed in distilled water, blotted dry and mounted.

As with all chromosome banding techniques, slides may respond differently to the staining procedure. However, altering the pH between 10.2 and 10.6 and the phosphate concentration between 0.2 and 0.4 M, could generally achieve satisfactory results.

The metaphase chromosomes of cells grown in the absence of BrdU are refractory to Giemsa staining with the present technique . After two rounds of replication in the presence of BrdU, the fully-substituted chromatid stains an intense magenta, whereas the half-substituted chromatid remains pale (Figure 13.). After three rounds of replication, chromosomes appear with both chromatids staining intensely.

This method of differentially staining sister chromatids was used because it was relatively quick and simple to perform, giving permanent preparations.

Figure 13. SISTER CHROMATID EXCHANGE



6.28.2 SLIDE SPREADING AND SCANNING

The same methods as used for conventional analysis were employed (6.27.1 and 6.27.3). Latt et al (1981) reported that a minimum of 25 cells should be scored per replicate and at least 50 per treatment. In this study, 60 cells were scored per replicate (120 per treatment). SCEs were expressed on a per metaphase basis as suggested by Latt et al (1981) and the number of chromosomes per cell was recorded.

6.28.3 PHOTOGRAPHY

6.28.31 FILM DEVELOPMENT

1. In complete darkness, the film negative was wound around the plastic spool of the light-tight negative holder.
2. In the light, negative developer was added and left for 6 minutes, during which the container was agitated.
3. The developer was poured off and Fixative added then left for 3 minutes with frequent agitation.
4. Tap water rinse for 30 minutes.
5. The film was removed from the container and spool, then hung to dry.

6.28.32 PRINT DEVELOPMENT

1. Following the exposure of the developing paper on the enlarger, the print was dipped into Paper Developer until an image appeared.
2. Stopbath of tap water.
3. The print was then dipped into Fixative and remained there for 10 minutes.
4. The prints were then placed in a water tank and washed for 30 minutes, then left to dry.

6.29 DNA SYNTHESIS

^{3}H- Thymidine incorporation into DNA measured quantitatively gives an indication of the comparable rate of DNA synthesis between two samples. In this study two such methods were employed, the latter being an improvement on the former, with regard to experimental accuracy.

The first method employed used whole EC cells which were rinsed to remove any ' hot ' media which had remained after incubation but before scintillation counting. The second revised method improved on this technique by breaking down the cell and nuclear membranes with sodium-dodecyl sulphate (SDS), thus allowing the nucleic acids to be thoroughly washed, preventing an inaccurate result due to trapped radioactivity in the cytoplasm and not incorporated into DNA.

METHOD 1.

1. On day 12 of the experiment, instructions 3. to 8. the Cell Culturing method was followed (see Section 6.20.1)

2. The supernatant was poured off and the pellet of cells gently resuspended in 1 ml of fresh complete medium, afterwhich, the tightly capped conical test tubes were vortexed inorder to thoroughly disperse the cells.

3. A cell count was performed on this cell suspension (Section 6.20.5.6),and the cell suspension diluted, if necessary, to contain 5×10^5 cells per ml.

4. The 1 ml of cell suspension containing 5×10^5 cells was then added to the old culture flasks now held in an upright position to reduce the surface area to 80 mm^2.

5. To each flask (control and treated) was added a futher 1 ml of complete fresh medium.

6. To each 2 ml in the old culture flask was added 1.0 μCi of ^3H- Thymidine, and the suspensions incubated for four hours in the dry incubator at 37°C.

7. To the culture flasks were added 50 ml of cold (4°C) PBS in an effort to terminate thymidine incorporation, and culture flasks were then stored overnight in the refrigerator (4°C for 14 hours).

8. The culture flasks were removed from the refrigerator and placed in an ice bath until the contents were emptied into the funnel of the millipore filtering system connected to a suction inlet at the water tap. The cells remained behind on the filter disc whilst the liquid was discarded into the flask below.

9. The old culture flask was rinsed with 20 ml of cold PBS and this suspension also slowly poured into the funnel.

10. The cells on the disc were then washed with 2 x 10 ml volumes of 10% TCA.

11. Two washes of absolute Methanol (2 x 20 ml) followed, to remove all traces of the TCA.

12. The glass-fibre discs were removed from the filter holder and put into the labelled scintillation vials then dried in a drying oven at 60°C for 1 hour.

13. To each vial was added 10 ml of scintillant and after 30 minutes, each sample was put through the Beckman Scintillation counting system.

METHOD 2.

1. On day 12 of the experiment, the 10 ml of old medium was removed from the culture flasks and replaced with 5 ml of ' hot ' media (2.5 μCi of ^{3}H- Thymidine) then re-incubated at 37°C for 4 hours.

2. At the end of incubation, the ' hot ' medium was removed and the cell layer rinsed three times with 5 ml of cold (4°C) PBS.

3. 2.0 ml of 0.5% SDS (containing 0.1% DNA) was added to the culture flasks which were then left for 5 minutes inorder to allow the cells to lyse.

4. 2 x 100 µl aliquots of the flask contents were removed for protein determinations (Lowry Method see section 6.29.2).

5. 2.0 ml of 20% TCA was added to the culture flasks and left for 5 minutes.

6. The contents were poured into the funnel of the millipore filtering system attached to a negative (suction) pressure source.

7. To the flasks was added 5 ml of 5% TCA, the contents then added to the funnel of the filtering system.

8. The filter discs were washed with a futher 10 ml of 5% TCA.

9. 2 x 10 ml washes of 96% Methanol (Absolute).

10. The filter disc with cell ' debris ' was then removed from the filtering system and put into the labelled scintillation vial (20 ml capacity) using small forceps, and the vials put in the dry oven at 60°C for 1 hour.

11. Dimilum-30 liquid scintillation cocktail with chemiluminescence inhibitor was added and liquid scintillation counting performed using a counter with an automatic quenching correction factor.

6.29.1 PROTEIN DETERMINATION : LOWRY METHOD (Lowry et al 1951).

The method involves the formation of a copper-protein complex in an alkaline solution. This complex then reduces a phosphomolybdie-phosphotungstate reagent (Folin), to yield a blue colour the intensity ofwhich is proportional to the amount of protein present which can in turn be related to the cell density.

' STANDARD CURVE '

1. In duplicate, microlitre amounts (20, 40, 60, 80, and 100 μl)of a 1 μg/μl solution of a serum albumin solution in 0.5% SDS were added to small plastic tubes.

2. These volumes were made up to 100 μl with 0.5% SDS (100 μl of SDS used as the blank).

3. 400 μl of distilled water was then added and 2 ml of solution X (see section 6.19).

4. 200 μl of Folin reagent was added and the samples mixed and left for 30 minutes before the absorbance measurements were made using a spectrophotometer at 750 nm.

6.29.2 DETERMINATION OF THE SAMPLE PROTEIN CONTENT

1. 2 x 100 µl aliquots of each treatment procedure and controls (Section 6.29.1 : Method 2, Instruction 4.) were placed in small plastic tubes to which was added 400 µl of distilled water.

2. 2 ml of Solution X was added to each tube.

3. 200 µl of Folin reagent was then added.

4. The samples were mixed thoroughly and left for 30 minutes before absorbance readings were determined at 750 nm using a spectrophotometer.

6.29.3 STATISTICAL ANALYSIS : DNA SYNTHESIS

The distribution - free or non parametric techniques were used here because conclusions could be drawn about results which required fewer qualifications than parametric testing. The former techniques of inference do not make numerous or stringent assumptions about a normal distribution or otherwise, of the population from which the sample was drawn.

Non parametric tests focus on the order of ranking of scores, not on their actual " numerical " values. Various ranking tests have been designed to test the significance of this type of information and the one used in this study to determine if the DNA synthesis results of one treatment were different from the results of another, is the Wilcoxon Signed - Rank Test.

This test was developed by Wilcoxon (1945), although it is sometimes called the Mann-Whitney Test. The particular procedure used here involved the ranking of unpaired measurements as follows:-

1) All observations of both treatments were put into a single array (with each group still being distinguishable from each other).
2) Ranks were then assigned to the combined array.
3) Finally, the sum of the ranks for each treatment was calculated, and when the value for the one treatment (usually the control) fell outside a specified range (Documenta Geigy, Statistical Tables), the null hypothesis of " No difference between groups " was rejected.

Table 12. ANALYSES PERFORMED

ANALYSES	EXPERIMENT A	B	C	D
Morphological Examination	+	+	+	+
Conventional Chromosome Analysis	+	+	+	+
Sister Chromatid Exchange Analysis	-	-	+	+
DNA Synthesis Analysis	-	+	+	+

+ = performed : - = not performed

Table 13. EXPERIMENTAL PLAN

KEY TO Table 13.

RA = Retinoic Acid

S-9 Mix = S-9 Mixture

DPH = Diphenylhydantoin

\+ = Performed

\- = Not Performed

* = Actual DPH values as measured by EMIT® (µg/ml) in prepared culture media.

** = Approximate amount of metabolite (µg/ml) added during media preparation.

Continued/...

Table 13. EXPERIMENTAL PLAN (DPH DOSAGES)

Code	TREATMENT	EXPERIMENT A	B	C	D
1	Control (no RA or drug)	+	+	+	+
2	RA (10^{-6}M)	+	+	+	+
3	S-9 Mix	+	+	+	-
4	RA (10^{-6}M) and S-9 Mix	+	+	+	-
5	Solvent only	+	+	+	-
6	DPH (High dose)	+ 492.6*	-	-	-
7	DPH (Intermediate dose)	+ 52.2*	+ 48.6*	+ 49.0*	+ 50.8*
8	DPH (Low dose)	+ 5.3*	-	-	-
9	DPH (High dose) and RA (10^{-6}M)	+ 516.0*	-	-	-
10	DPH (Intermediate dose) and RA (10^{-6}M)	+ 54.0*	+ 53.4*	+ 45.6*	+ 49.2*
11	DPH (Low dose) and RA (10^{-6}M)	+ 4.5*	-	-	-
12	DPH (High dose) and S-9 Mix	+ 504.2*	-	-	-
13	DPH (Intermediate dose) and S-9 Mix	+ 48.4*	+ 52.8*	+ 54.0*	+ 50.2*
14	DPH (Low dose) and S-9 Mix	+ 4.8*	-	-	-
15	DPH (High dose), RA (10^{-6}M) and S-9 Mix	+ 486.0*	-	-	-
16	DPH (Intermediate dose), RA (10^{-6}M) and S-9 Mix	+ 48.2*	+ 48.8*	+ 52.2*	+ 50.6*
17	DPH (Low dose), RA (10^{-6}M) and S-9 Mix	+ 5.9*	-	-	-
18	DPH m-hydroxy metabolite	-	-	-	+ 50.0**
19	DPH m-hydroxy metabolite and RA (10^{-6}M)	-	-	-	+ 50.0**
20	DPH p-hydroxy metabolite	-	-	-	+ 50.0**
21	DPH p-hydroxy metabolite +RA (10^{-6}M)	-	-	-	+ 50.0**

A summary of the culture programme involved in Experiments A, B, C, and D now follows. Each experiment was over a 12 day period, with chronic drug exposire for 5 days (days 7 - 12).

Table 14 . CULTURE PROGRAMME FOR EXPERIMENTS A,B,C AND D.

DAY	PROCEDURE
1	Cells plated—initial cell density per flask:- Experiment A = 10^7 cells " B = 10^7 cells " C = $5x10^6$ cells " D = $5x10^6$ cells
2	Media change
3	Subculture
4 *	Media change
5*	Subculture
6*	Media change
7*	Subculture— Drug added (where necessary)
8	Media change
9	Media change
10	Media change— BrdU added for SCE analysis
11	Media change
12*	Culture termination

Note: Morphological examination using phase contrast microscopy was performed daily to check on cell culture development and photographs taken on days indicated *.

Number of culture flasks employed:-

i) Morphological observations- made on all culture flasks.

ii) Conventional chromosome analysis and SCE- were performed in duplicate.

iii) DNA synthesis measurments were made on six flasks for each treatment.

The p-hydroxy metabolite was in short supply, and so a single flask was used for ii) and three flasks were used for iii).

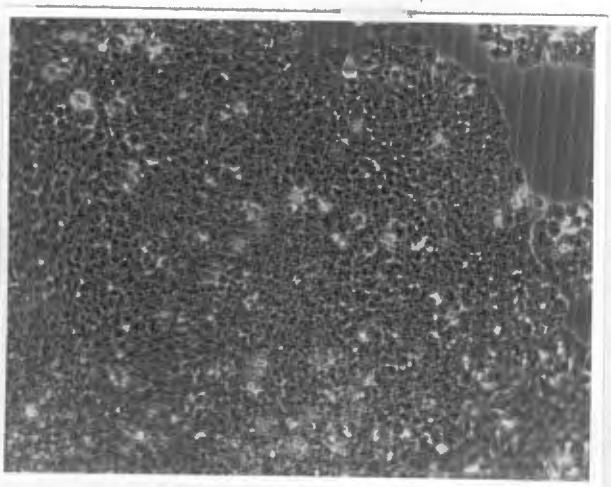
CHAPTER 7.

7.0 RESULTS

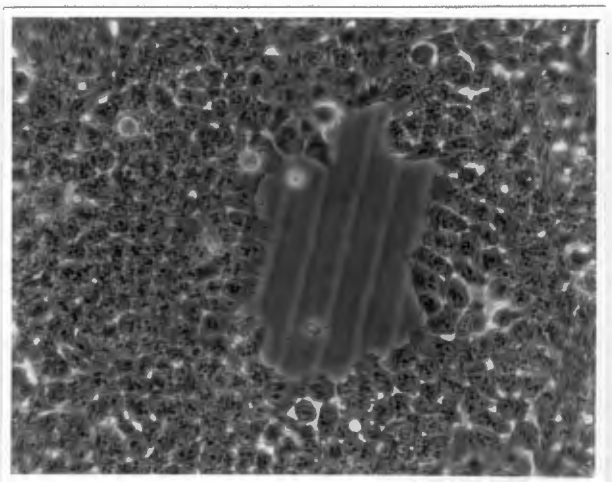
7.1 NORMAL MORPHOLOGY OF PC13 EC CELLS IN CULTURE

Cell cultures of PC13 EC cells differentiate minimally, although occasional Endoderm-like, Fibroblast-like, and Glial-like cells may be seen but routinely represent less than 1% of the total population. Phase contrast microscopy of the normal cell cultures reveals (Figure 14 a. b. and c.) the general morphology of the PC13 EC cells, which is futher supported by a photomicrograph (Figure 15.) of a histologically processed slide preparation of the fixed cells. The cells were seen to be small (about 10 μm), rounded, with scant cytoplasm and often indistinct borders, giving the appearance of a syncytium. These cells usually have a large central nucleolus and little in the way of cytoplasmic structures. Mitotic figures were common (Figure 15. arrows).

Figure 14. NORMAL CELL MORPHOLOGY : CELL CULTURES



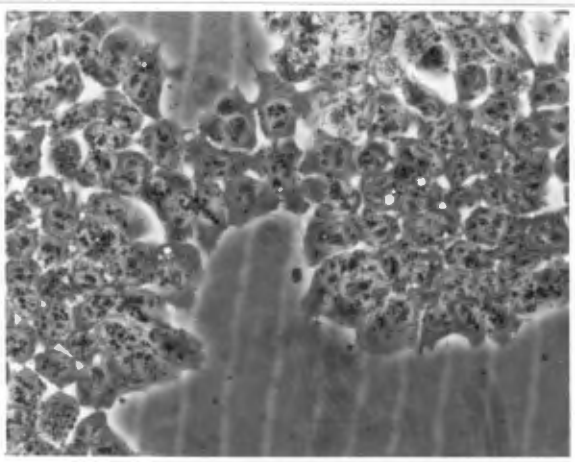
(x 80)



(x 250)

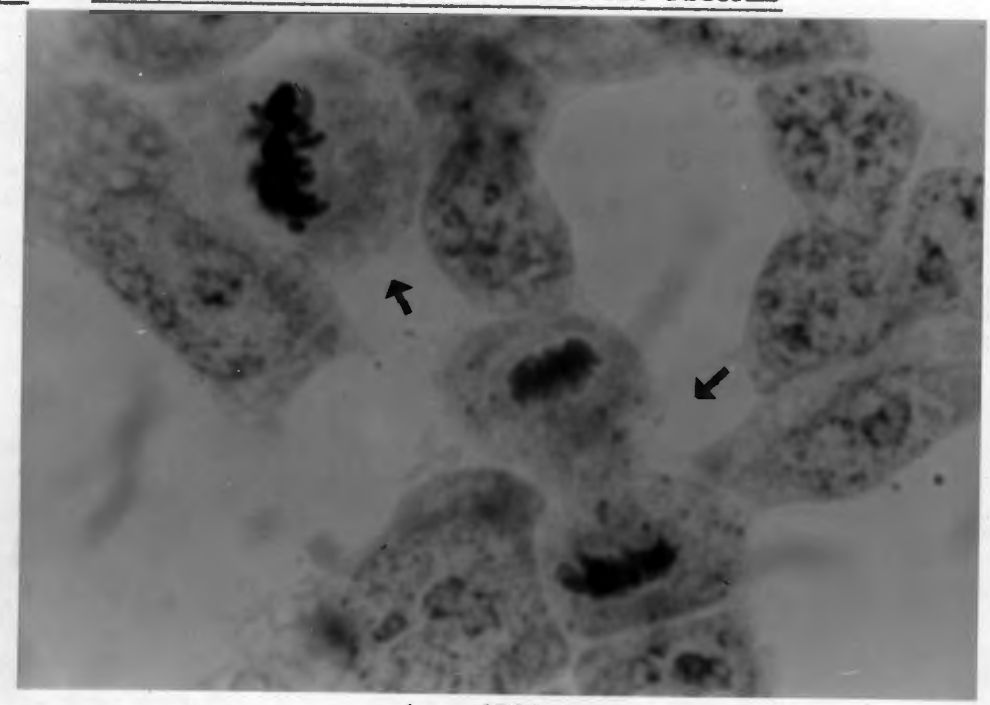
Figure 14. Continued/...

c.



(x 500)

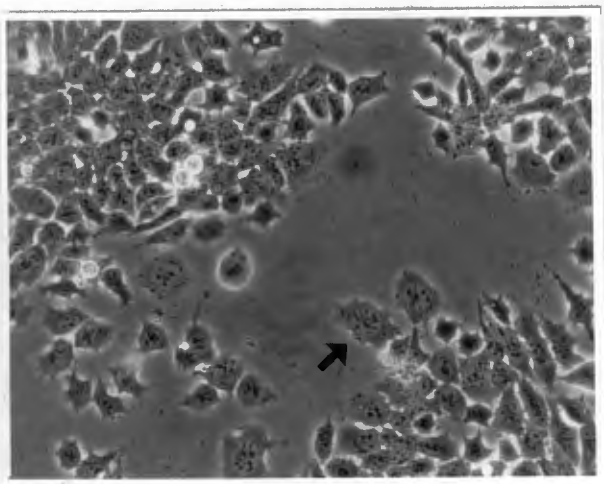
Figure 15. NORMAL CELL MORPHOLOGY AND MITOTIC FIGURES



(x 1500)

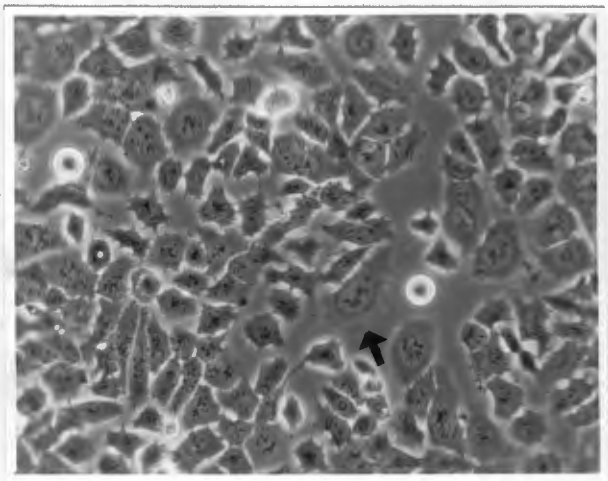
Figure 16. LOW CELL DENSITY PLATING (7 x 10^5 CELLS/ FLASK) AND THE INDUCTION OF CELL DIFFERENTIATION

a. DMEM : Day 3.



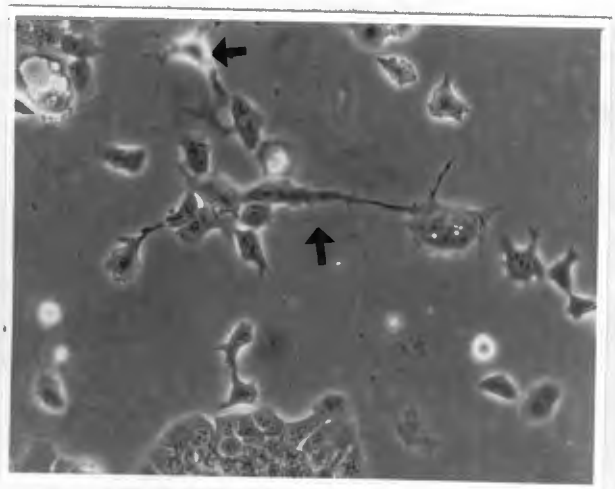
(x 250)

b. EMEM : Day 3.



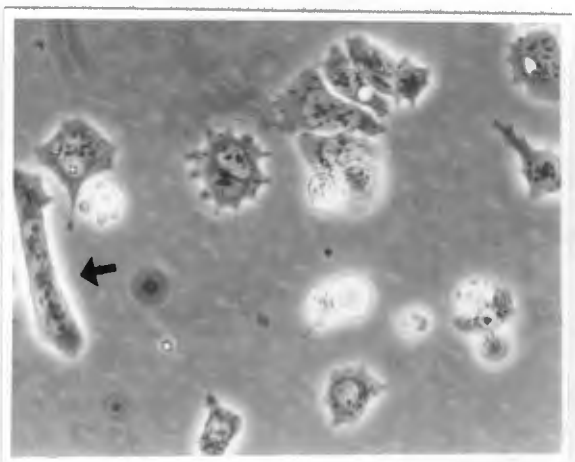
(x 250)

c. DMEM : Day 5.



(x 250)

d. EMEM : Day 5.



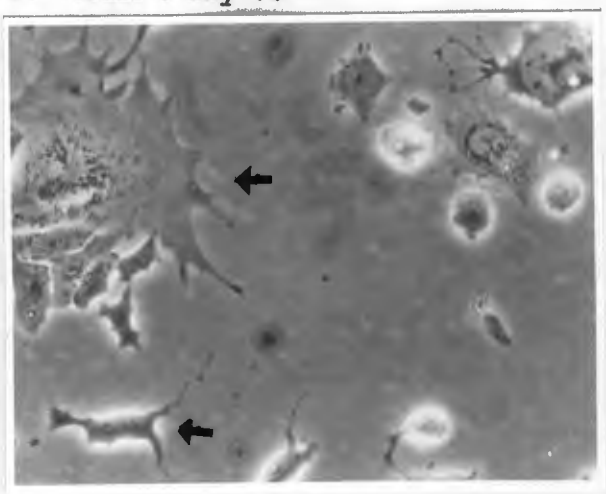
(x 250)

Figure 16. Continued/...

e. DMEM : Day 7.

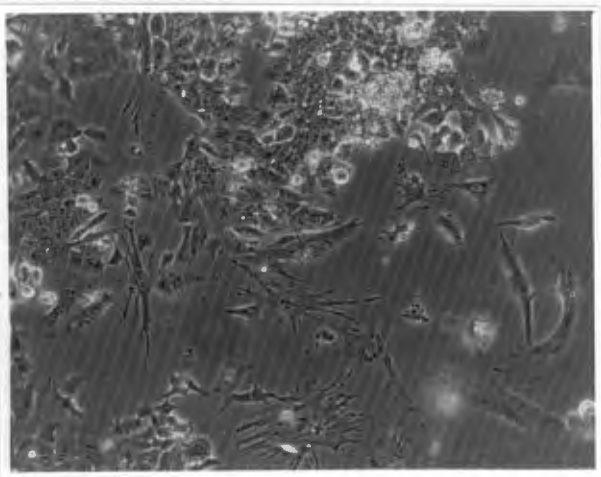
(x 250)

f. EMEM : Day 7.



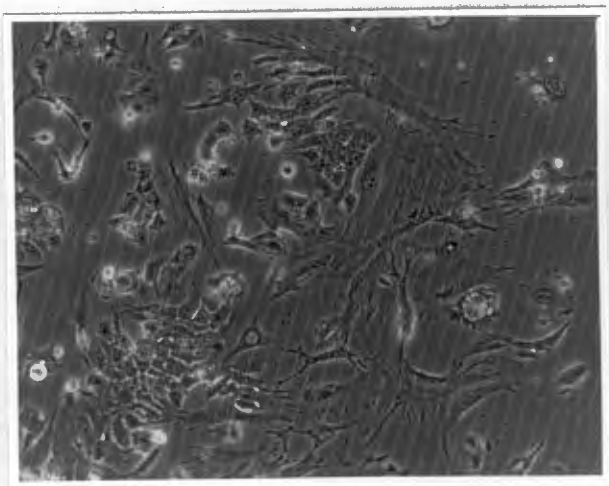
(x 250)

g. DMEM : Day 21.



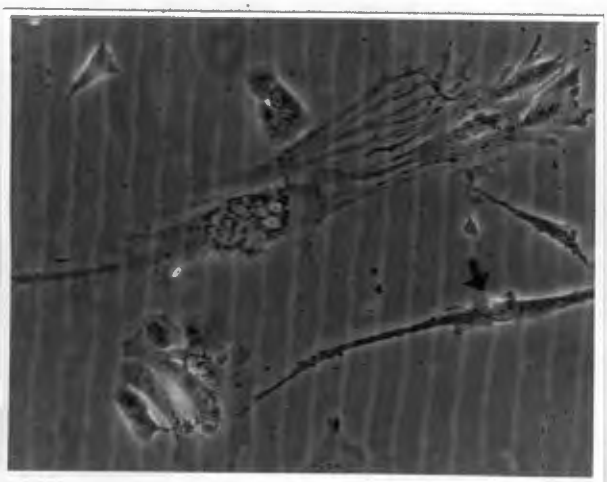
(x 80)

h. EMEM : Day 21.



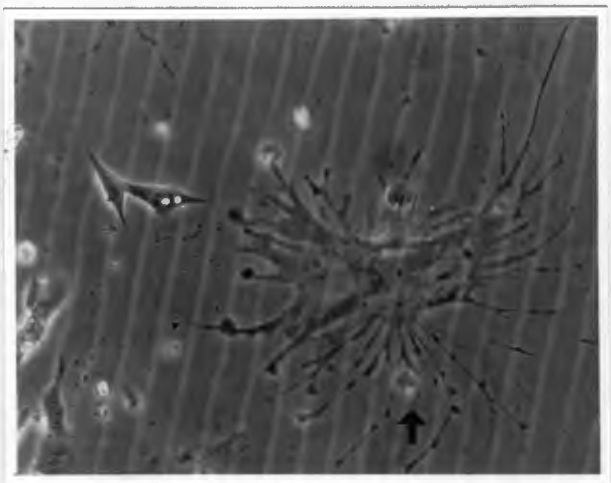
(x 80)

i. DMEM : Day 21.



(x 250)

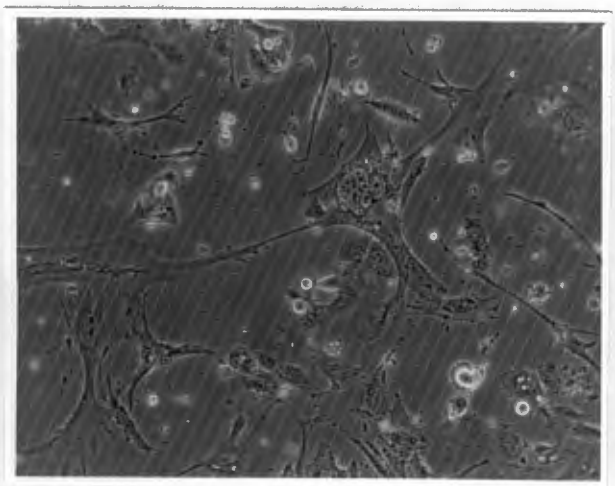
j. EMEM : Day 21.



(x 250)

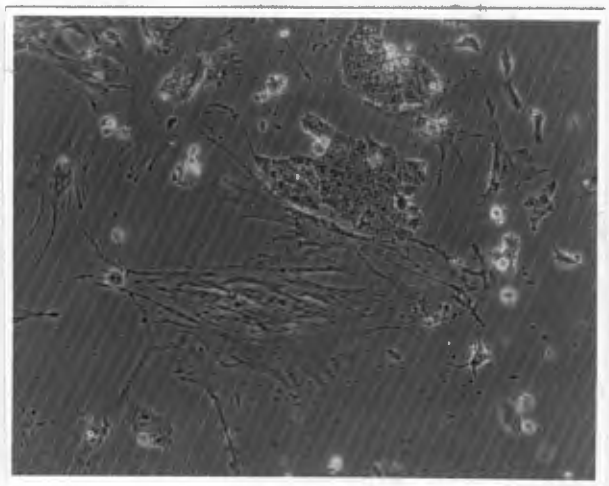
Figure 17. LOW CELL DENSITY PLATING (5 x 10^5 CELLS/ FLASK) AND THE INDUCTION OF CELL DIFFERENTIATION

a. DMEM : Day 21.



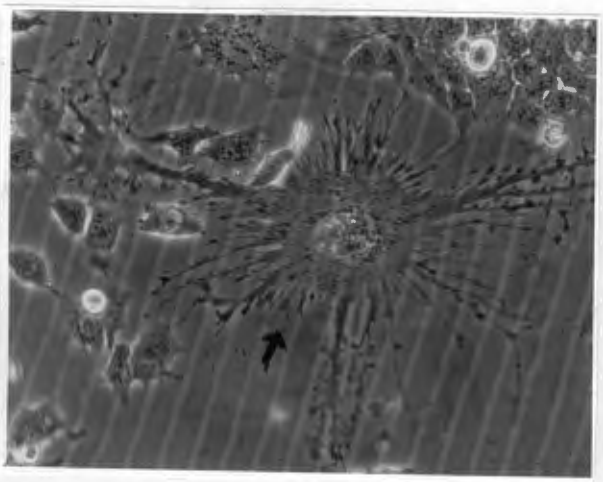
(x 80)

b. EMEM : Day 21.



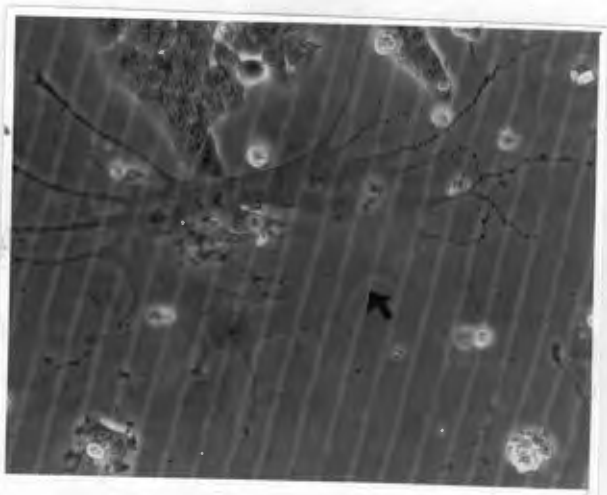
(x 80)

c. DMEM : Day 21.



(x 250)

d. EMEM : Day 21.



(x 250)

7.2 CELL DENSITY AND DIFFERENTIATION INDUCTION

In an attempt to culture PC13 EC cells at lower cell densities (initial cell densities of 5×10^5 and 7×10^5 cells per flask) in both DMEM and EMEM culture media so that growth could be observed for a longer period before confluency, the phenomenom of differentiation was observed (Figure 16. a. b. c. d. e. f. g. h. i. and j. and Figure 17. a. b. c. and d.). Cell culture plated initially with 10^6 cells per flask retained the normal growth pattern and did not differentiate as such.
With initial density 7×10^5, the cells did not proliferate very rapidly (when compared with controls) due to the induction of differentiation and when the cultures were examined on Day 21, the lower cell density cultures had become well differentiated with mainly Fibroblast-like cells (Figure 16. i. arrow) and Giant Multiprocessed (Neuron-like) cells (Figure 16 j. arrow).
On Day 3 of the experiment, the differentiating cells took the form of small granular Endoderm-like cells(Figure 16 a. and b. arrows). By Day 5, Fibroblast-like cells could be seen (Figure 16 c. and d. arrows) and on Day 7 the large Endoderm-like cells were apparent (Figure 16. e. and f.) with Glial-like cells (Figure 16 f. lower arrow).
These observations were also observed when an initial cell density of 5×10^5 cells per culture flask was plated (Figure 17.) and by day 21, very large multiprocessed Neuronal-like cells were present (Figure 17 c. and d. arrows). Results were the same for both types of culture media employed.

7.3 DIFFERENTIATION INDUCTION WITH RETINOIC ACID (10^{-6}M)

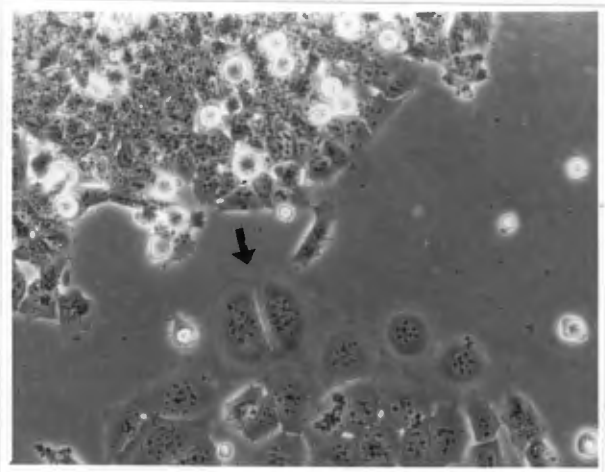
The PC13 EC cells in cell culture normally tend to form sheets of closely adherent polygonal cells and so have been termed Epithelial- like due to their mosaic-like appearance. When cell cultures become confluent, they lose their monolayered appearance, with the cells having a tendency to pile up as there is no contact inhibition, a phenomenom associated with teratocarcinoma cells.

Following the application of 10^{-6}M retinoic acid to PC13 stem cell cultures, small granular Endoderm-like cells began to to appear by Day 2 and were very apparent by Day 4 (Figure 18. a. and b. arrows). These early cells seemed to become replaced by two main cell types: Glial-like cells which appeared after about 5 days (Figure 19 a. and b. arrows) becoming more differentiated with time (Figure 19 c. and d.Day 6. arrows), also Fibroblast-like cells were observed (Figure 20 a. and b. Day 8. arrows) which seemed to develop into or become intimately involved with the larger Endoderm-like cell type. These larger cells may fail to develop the large cell processes and form the large Squamous epithelium-like cell type (Figure 21 a. and b. Day 14.) The cell in Figure 21 a. exhibits the " stress fibres " mentioned in Section 6.21.2 and Figure 21 b. illustrates the " thread-like extentions " also mentioned. Alternatively, the larger Endoderm-like cell type may go on to develop into the large multiprocessed neuronal-like cell type (Figure 22 a. b. c. and d. Day 18). The elongated neuronal type cells usually make contact with many neighbouring cells (Figure 22. a. and b. arrows).

Figures 18. to 24. are photographs representative of similar morphology observed in 8 replicate culture flasks over a period of 18 days. Initial cell density was 10^6 cells per culture flask.

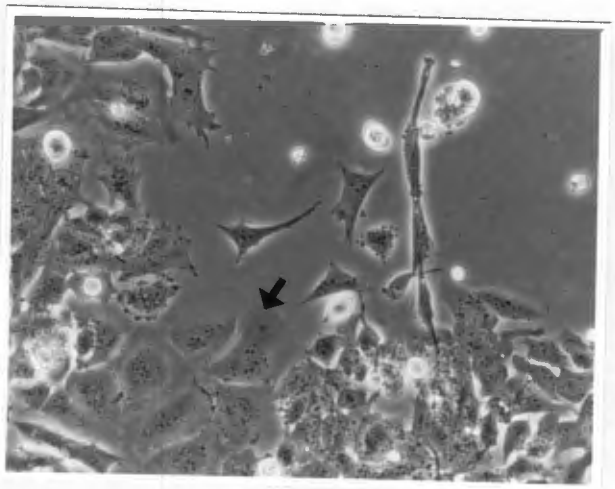
Figure 18. SMALL GRANULAR ENDODERM-LIKE CELLS

a. DMEM : Day 4.



(x 250)

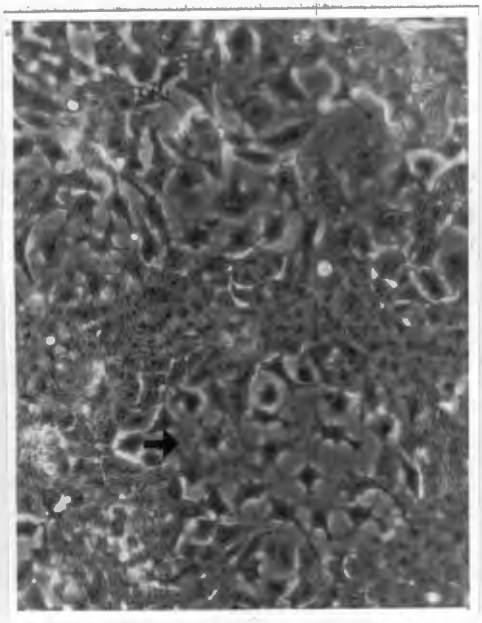
b. EMEM : Day 4.



(x 250)

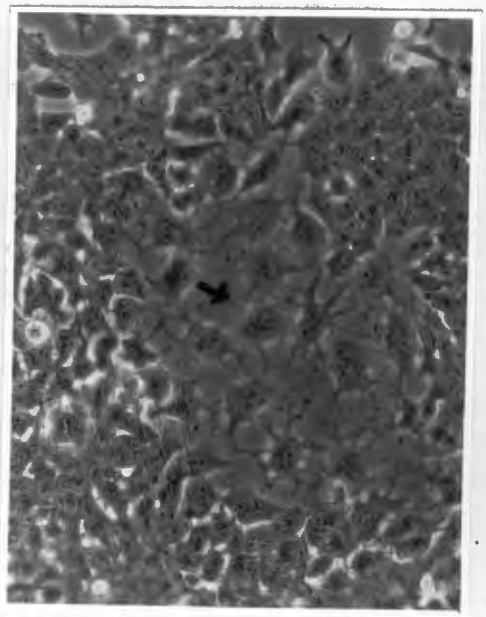
Figure 19. GLIAL - LIKE CELLS

a. DMEM : Day 5.



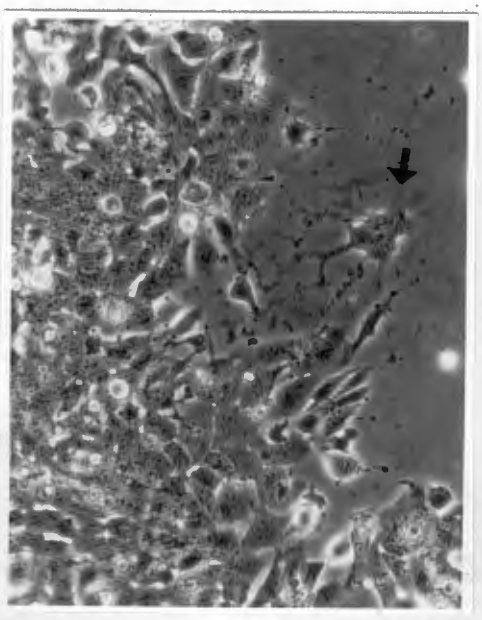
(x 250)

b. EMEM : Day 5.



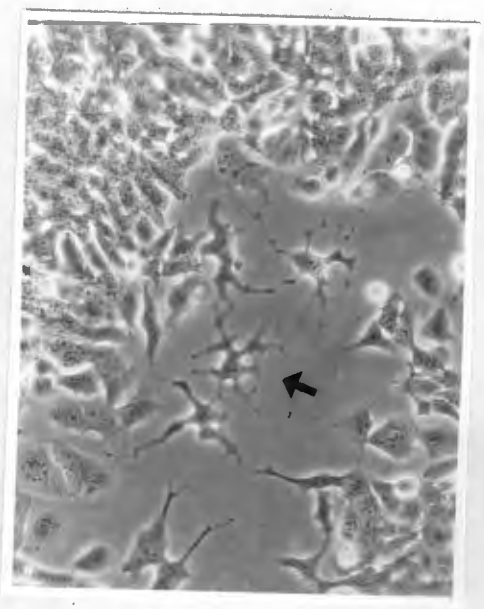
(x 250)

c. DMEM : Day 6.



(x 250)

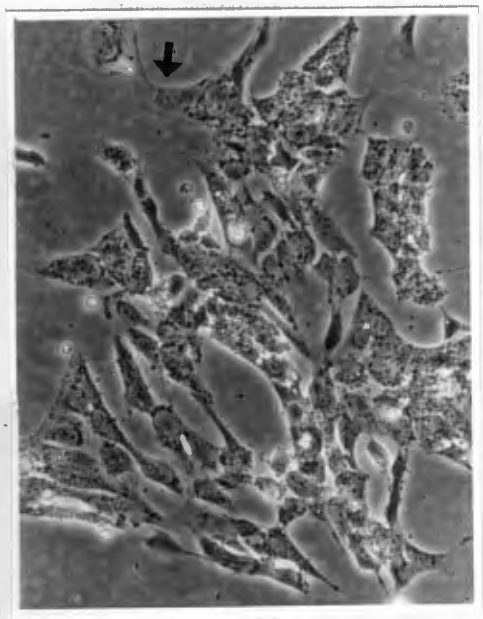
d. DMEM : Day 6.



(x 250)

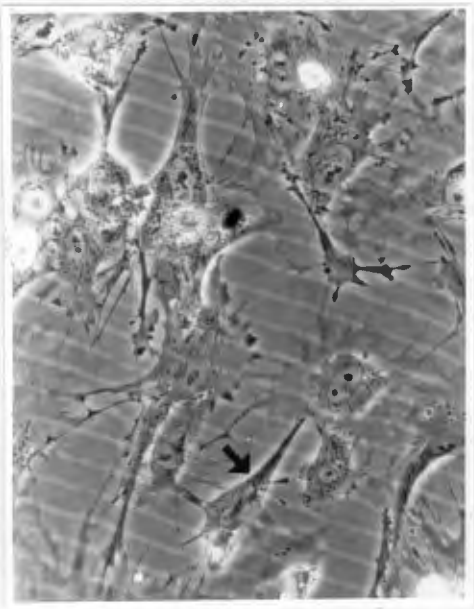
Figure 20. FIBROBLAST - LIKE CELLS

a. DMEM : Day 8.



(x 250)

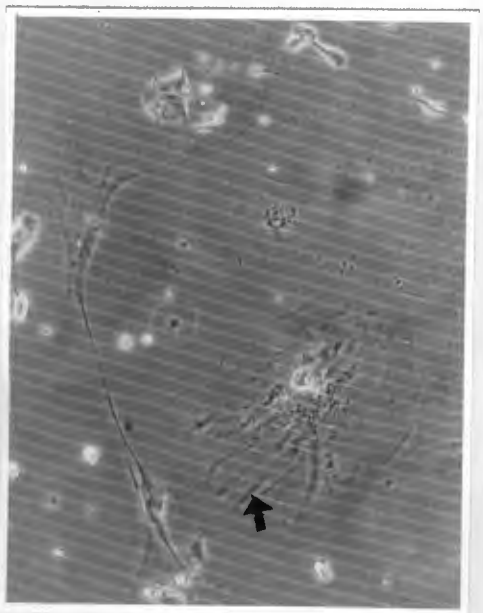
b. EMEM : Day 8.



(x 250)

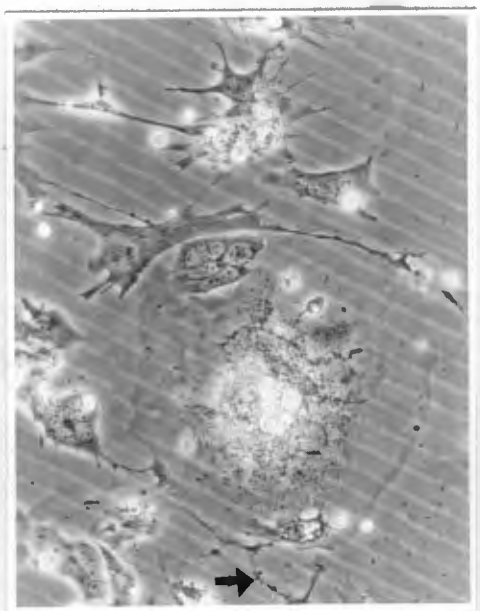
Figure 21. LARGE ENDODERM - LIKE CELLS

a. DMEM : Day 14.



(x 80)

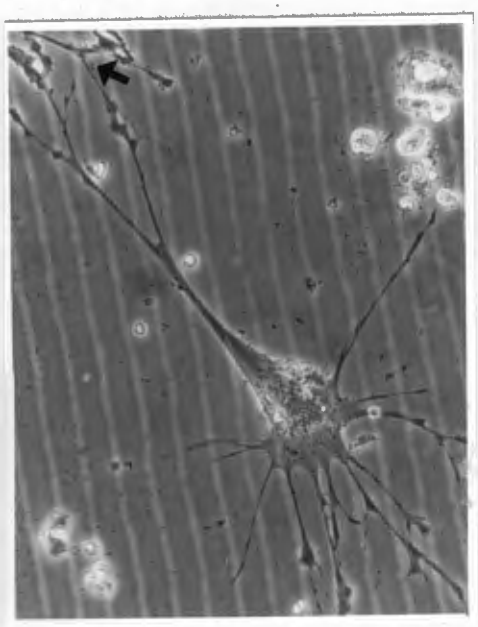
b. EMEM : Day 14.



(x 250)

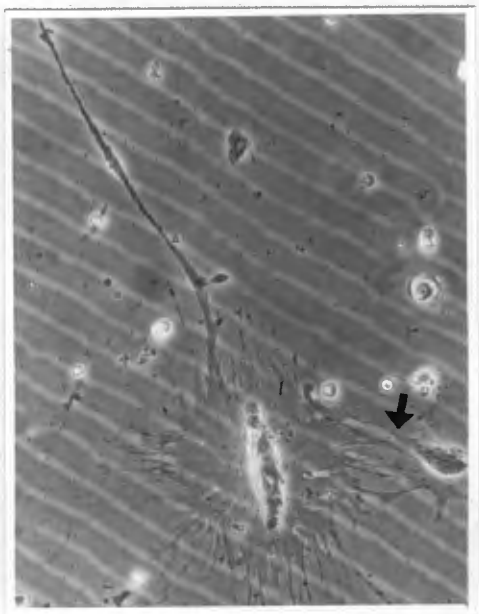
Figure 22. LARGE MULTIPROCESSED NEURONAL - LIKE CELLS

a. DMEM : Day 18.



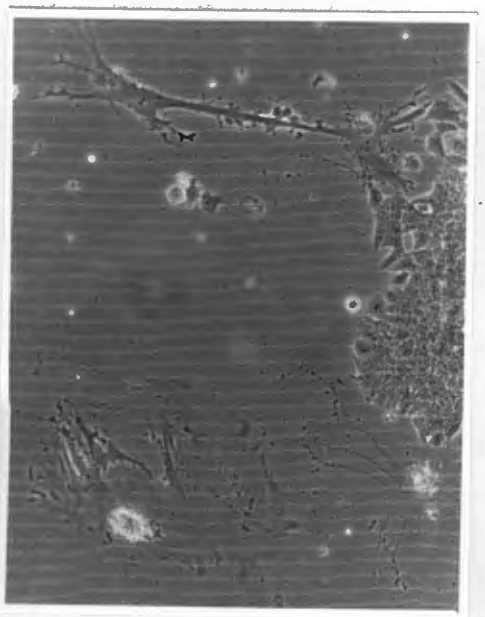
(x 250)

b. EMEM : Day 18.



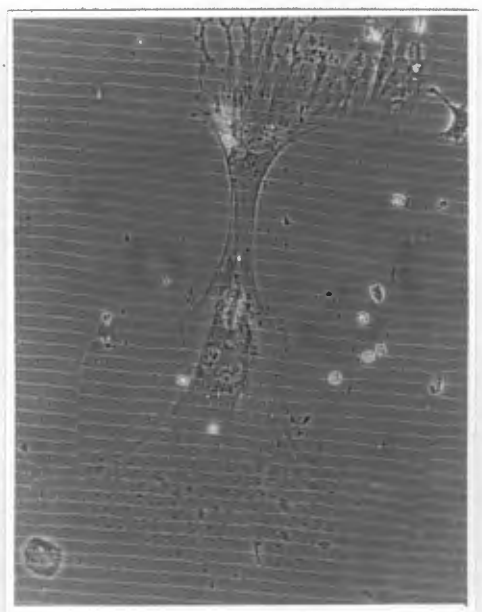
(x 250)

c. DMEM : Day 18.



(x 80)

d. EMEM : Day 18.

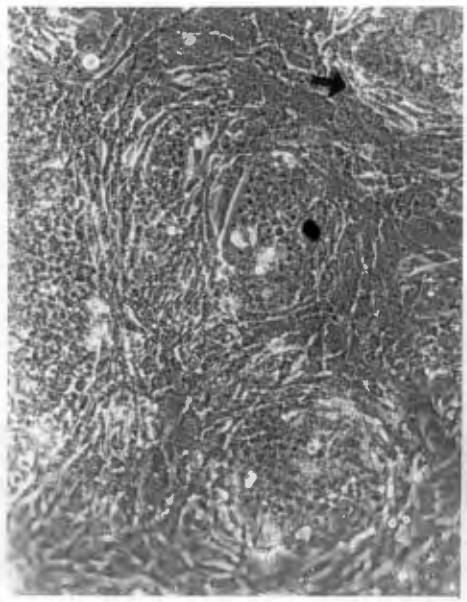


(x 80)

The Fibroblast-like cells formed distinct boundaries around the undifferentiated EC cells which tended to pile up (Figure 23 b. arrow) due to lack of contact inhibition. The junction between both cell types is indicated in Figures 23a. and 24. by the black arrows.

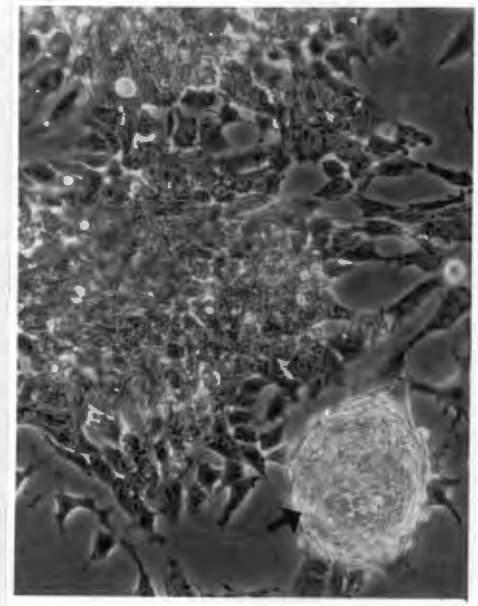
Figure 23. JUXTAPOSITION OF UNDIFFERENTIATED AND DIFFERENTIATING PC13 EC CELLS IN CULTURE

a. DMEM : Day 8.



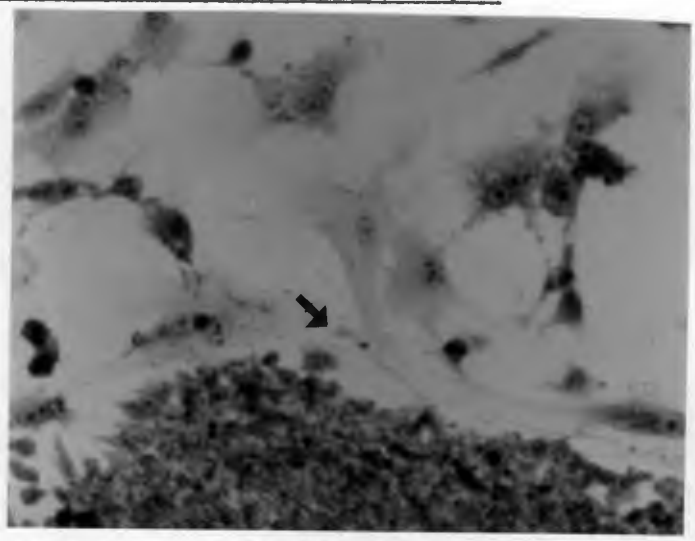
(x 80)

b. EMEM : Day 8.



(x 250)

Figure 24. HISTOLOGICAL PREPARATION : Day 8.



(x 200)

7.4 EXPERIMENTS A. B. C. AND D. : COMBINED TREATMENTS

For those cell cultures requiring differentiation induction, (Table 13.), there was the treatment of cultures with 10^{-6}M retinoic acid for a period of seven days, followed by specific culture treatments (for example, drug added alone, drug added with retinoic acid, drug added with S-9 mixture) for 5 days, until Day 12 of the experimental run onwhich cell cultures were terminated and analyses performed (Table 12.).

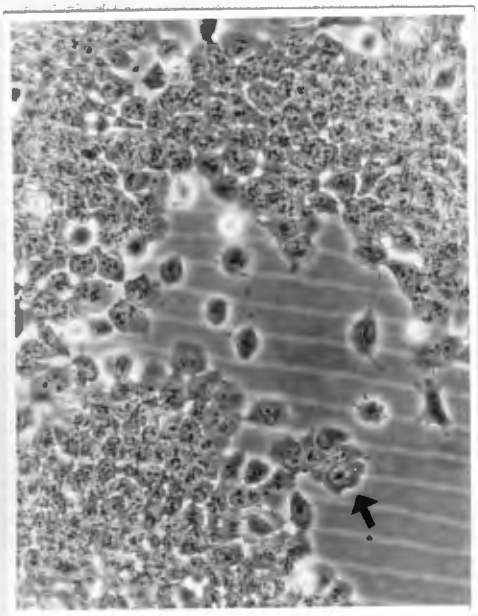
The medium used for these Experiments was DMEM because the cells grew well in this medium which did not tend to become acidic (indicated by phenyl red colour change to yellow) quite as readily as EMEM under the same conditions, this was a reflection on the actual media composition (Table 10.).

In order to achieve optimum cell culture growth, cultures were subcultured on Days 3. 5. and 7. This necessitated the use of an initial cell density of 10^{7}cells per flask (Experiments A. and B., later revised to 5 x 10^{6} cells per flask (Experiments C. and D.) inorder to overcome problems with low mitotic index due to confluency.

The morphological results for Experiments A. and B. were very similar throughout the experimental run and therefore only one photograph has been included to depict the observations made, this also applies to Experiments C. and D.

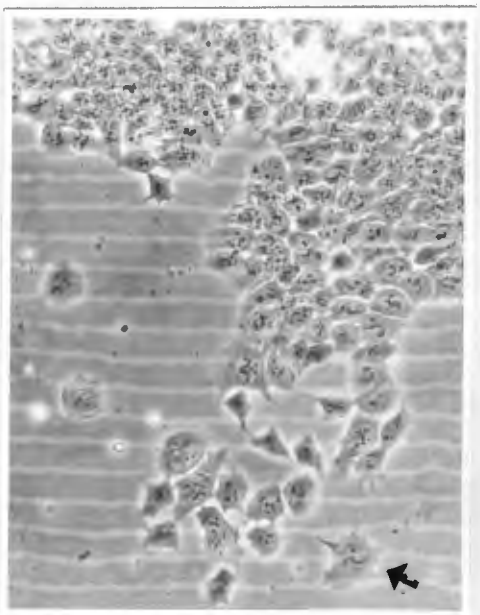
Figure 25. CELL MORPHOLOGY IN EXPERIMENTS A. B. C. AND D.
(Pre-treatment with Retinoic acid at 10^{-6}M for those treatments requiring differentiating cells - refer to Table 13.)

a. Experiments A. and B.
Day 4.



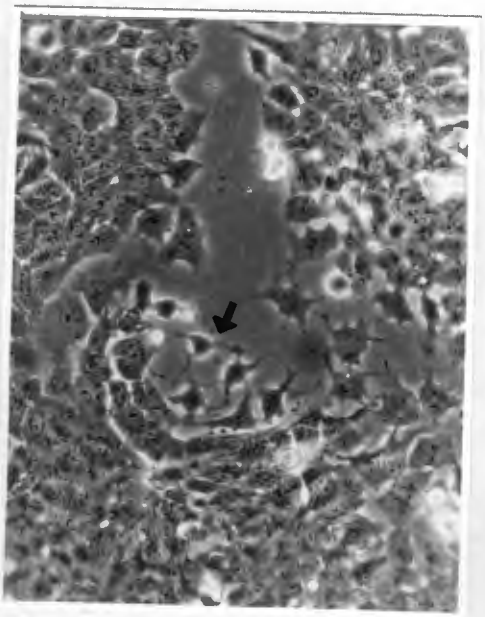
(x 250)

b. Experiments C. and D.
Day 4.



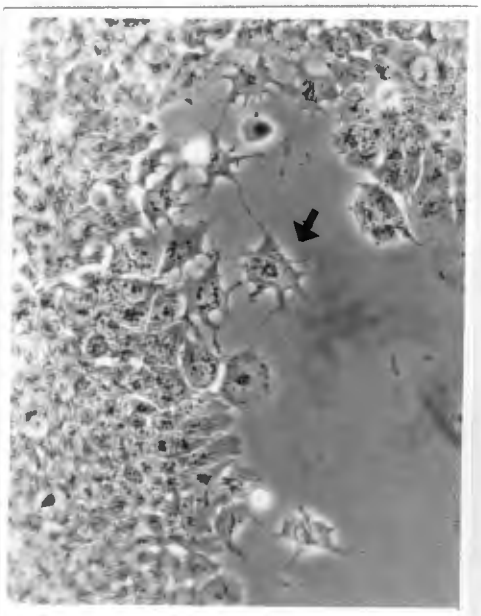
(x 250)

c. Experiments A. and B.
Day 5.



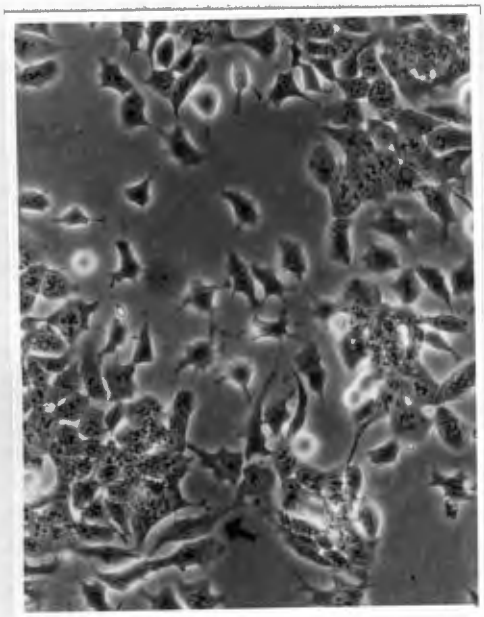
(x 250)

d. Experiments C. and D.
Day 5.



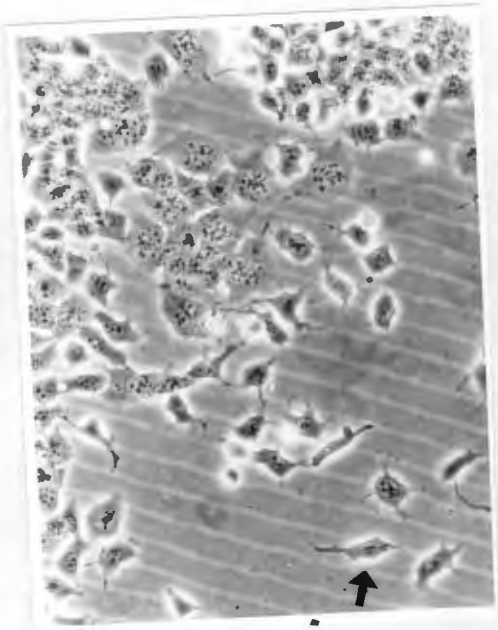
(x 250)

e. Experiments A. and B.
Day 6.



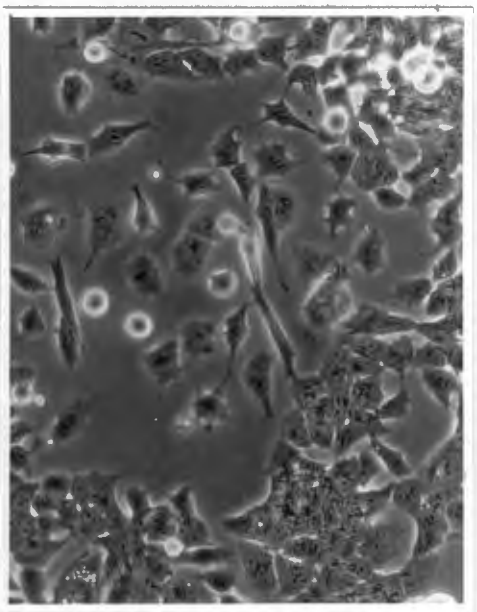
(x 250)

f. Experiments C. and D.
Day 6.



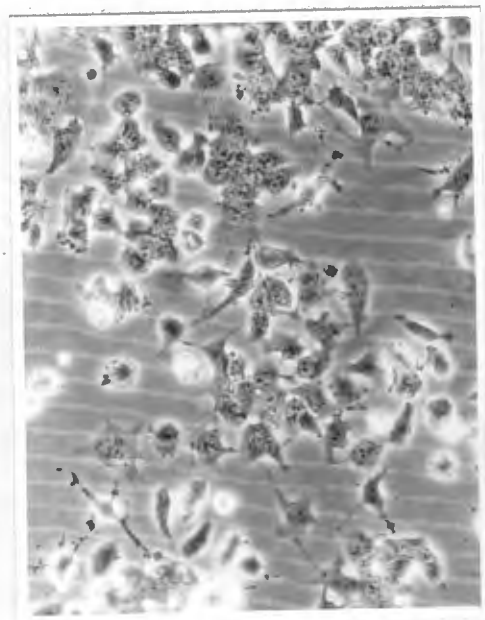
(x 250)

g. Experiments A. and B.
Day 7.



(x 250)

h. Experiments C. and D.
Day 7.



(x 250)

Cell morphology did not change from normal until about Day 3. and the small granular Endoderm-like cells became more apparent by Day 4. (Figure 25.a. and b. arrows). By Day 5. the Glial-like cells were observable (Figure 25 c. and d.) and Fibroblast-like cells by Day 6. (Figures 25 e. and f. arrows). All the aforementioned cell types and the undifferentiated EC cells were observed in the cell cultures on Day 7. (Figures 25 g. and h.) when the specific treatments were applied. The relative proportions of the Undifferentiated and Differentiating cell types were estimated using the ' point counting method ' mentioned in Section 6.21.2.

Table 15. PERCENTAGES OF CELL DIFFERENTIATION AFTER THE INDUCTION OF DIFFERENTIATION WITH 10^{-6}M RETINOIC ACID TREATMENT IN EXPERIMENTS A. B. C. AND D. (Estimations made on Day 7)

EXPERIMENT	Number of " hits " out of 121 points		% DIFF**
	EC*	DIFF**	
A.	35	86	41
B.	32	90	43
C.	28	93	50
D.	24	97	54
			47 (Average)

Note: Results are of an average of data obtained on 8 culture flasks

* = Undifferentiated PC13 EC cells

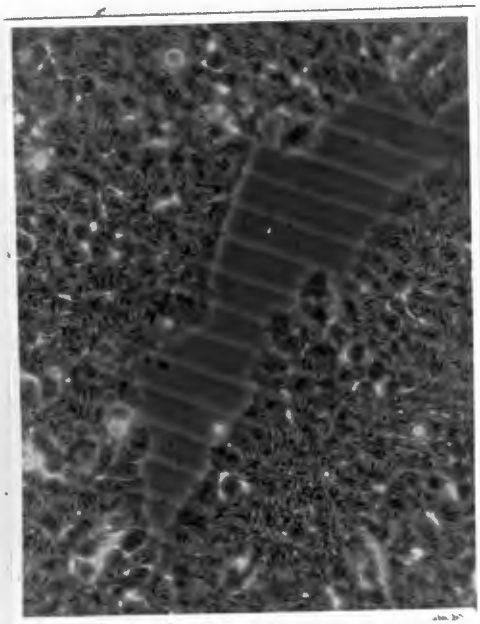
** = Differentiating PC13 EC cells

Refer to Section 6.21.2 for formula.

Photographs taken on Day 12, depicting the morphological appearance of cells in culture for all treatments in Experiments A/B and C/D now follows where * refers to approximate DPH dosages (see Table 13.) :-

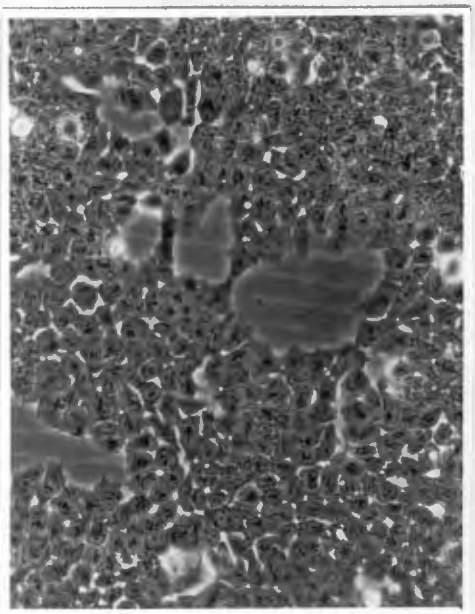
Figure 26. CONTROL (No Retinoic acid or drug present)

a. Experiments A/B.



(x 250)

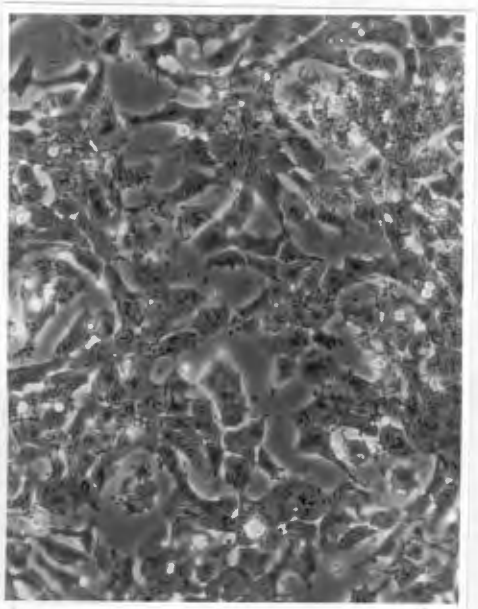
b. Experiments C/D.



(x 250)

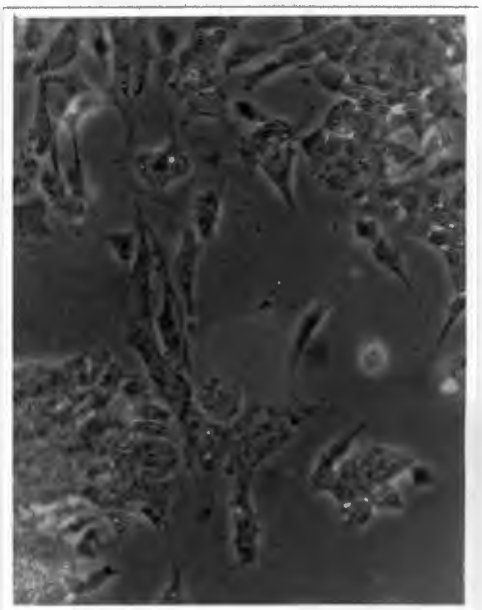
Figure 27. RETINOIC ACID (10^{-6}M)

a. Experiments A/B.



(x 250)

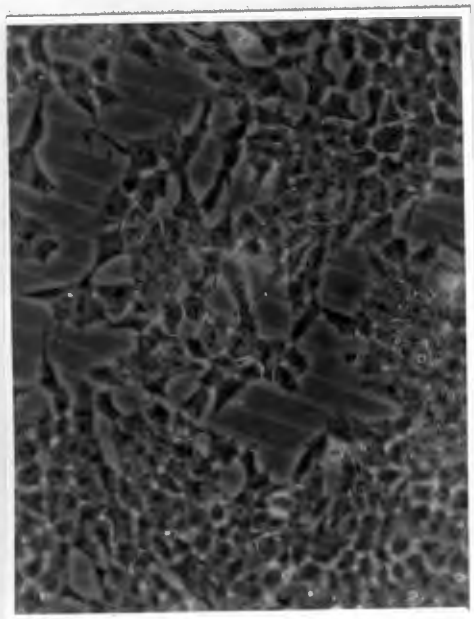
b. Experiments C/D.



(x 250)

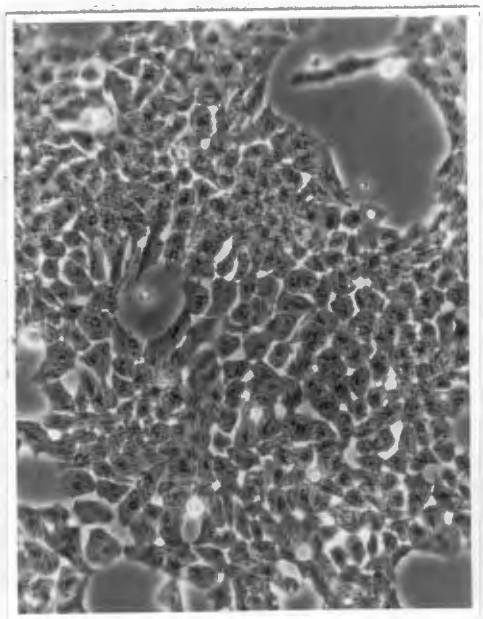
Figure 28. S-9 MIXTURE

a. Experiments A/B.



(x 250)

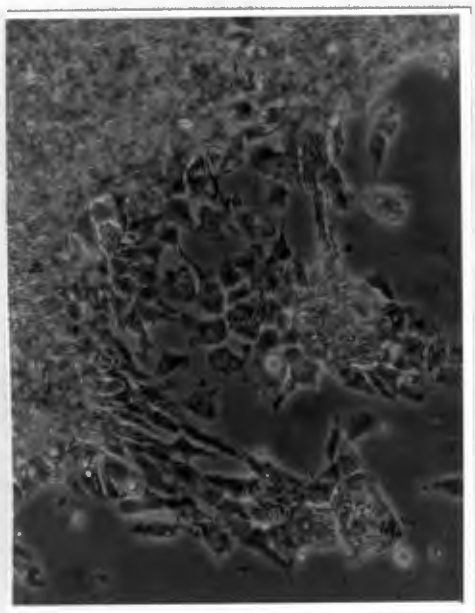
b. Experiment C.



(x 250)

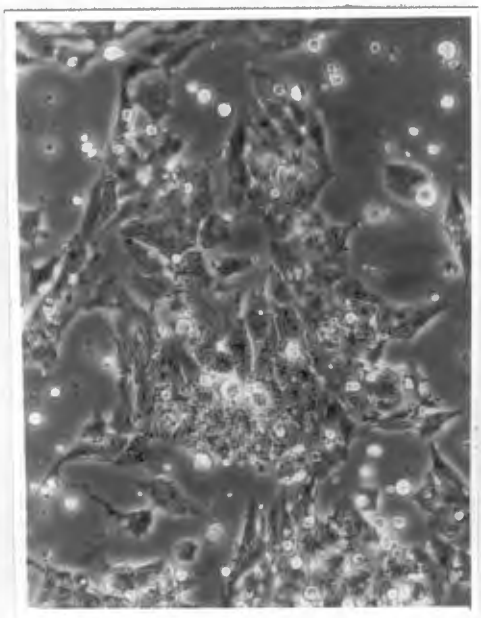
Figure 29. RETINOIC ACID (10^{-6}M) AND S-9 MIXTURE

a. Experiment A/B.



(x 250)

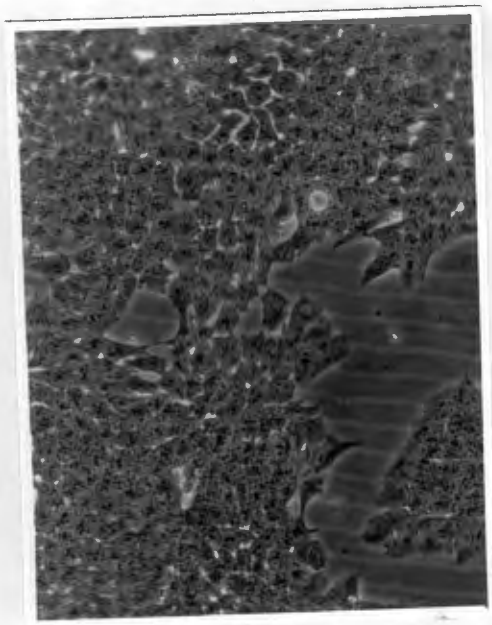
b. Experiment C.



(x 250)

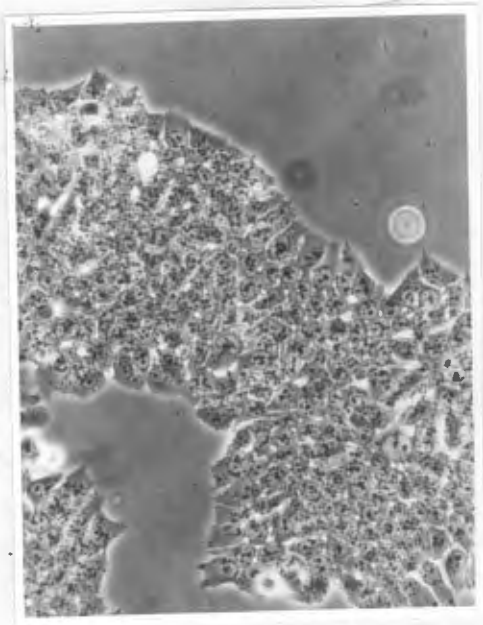
Figure 30. SOLVENTS

a. Experiments A/B.



(x 250)

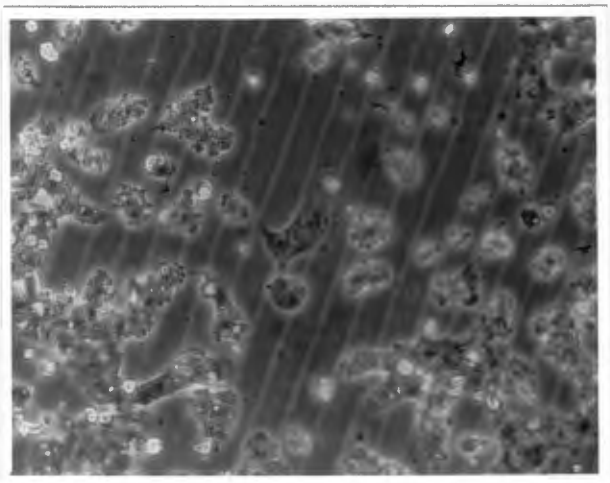
b. Experiments C.



(x 250)

Figure 31. DPH (500 μg/ml *)

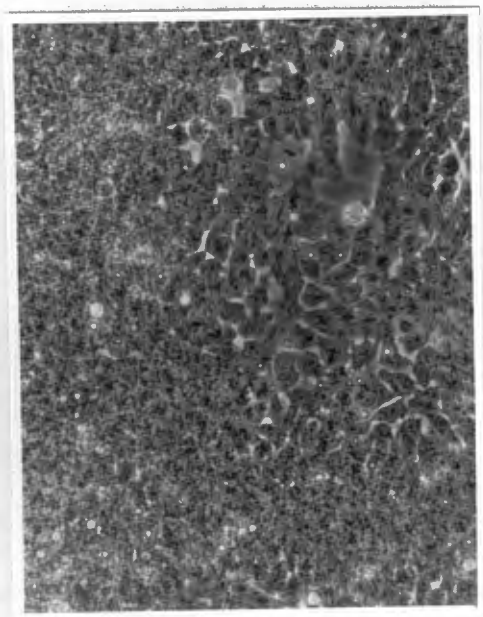
a. Experiment A.



(x 250)

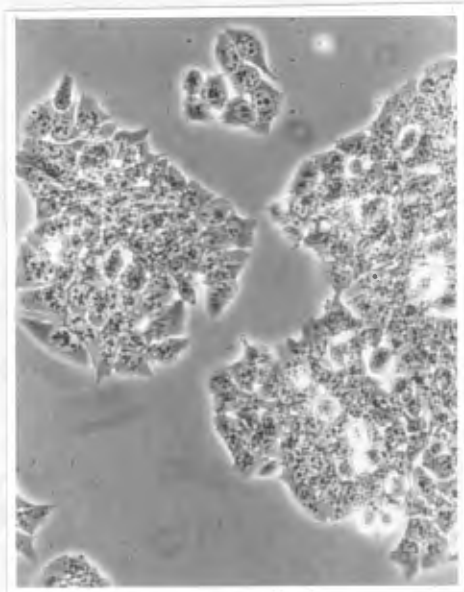
Figure 32. DPH (50 μg/ml *)

a. Experiments A/B.



(x 250)

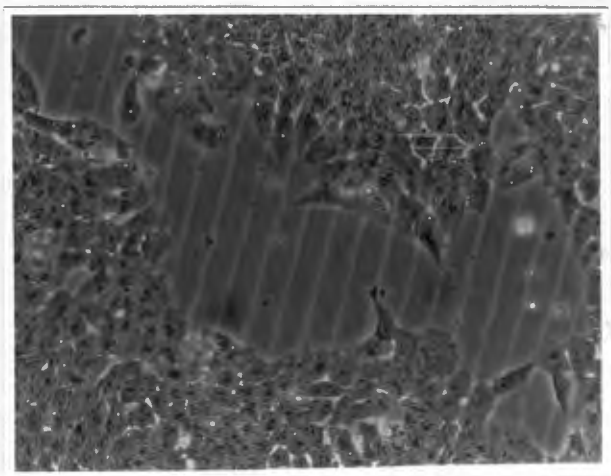
b. Experiments C/D.



(x 250)

Figure 33. DPH (5 μg/ml *)

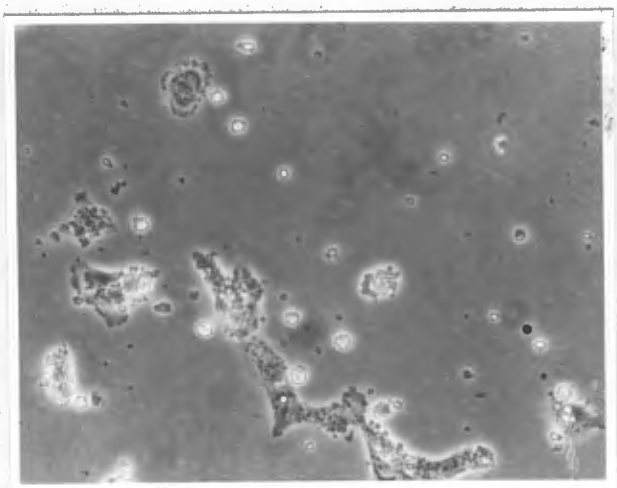
a. Experiment A.



(x 250)

Figure 34. DPH (500 μg/ml *) AND RETINOIC ACID (10^{-6}M)

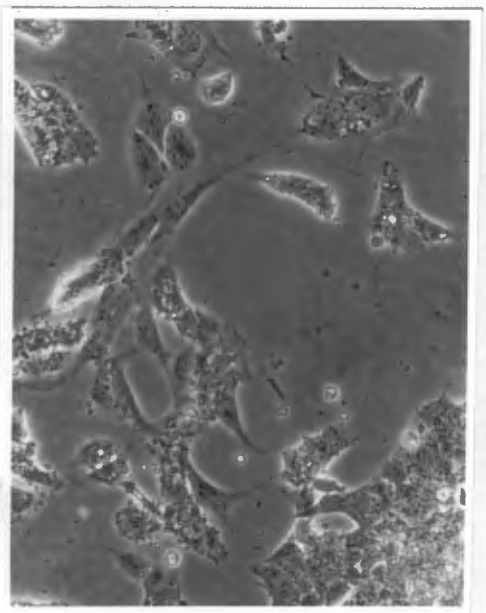
a. Experiment A.



(x 250)

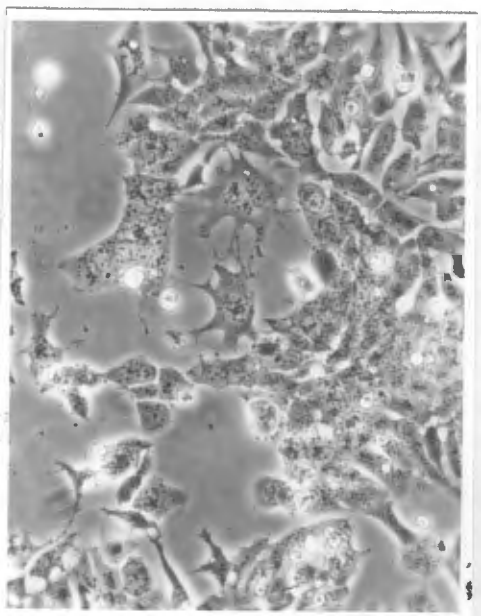
Figure 35. DPH (50 μg/ml *) AND RETINOIC ACID (10^{-6}M)

a. Experiments A/B.



(x 250)

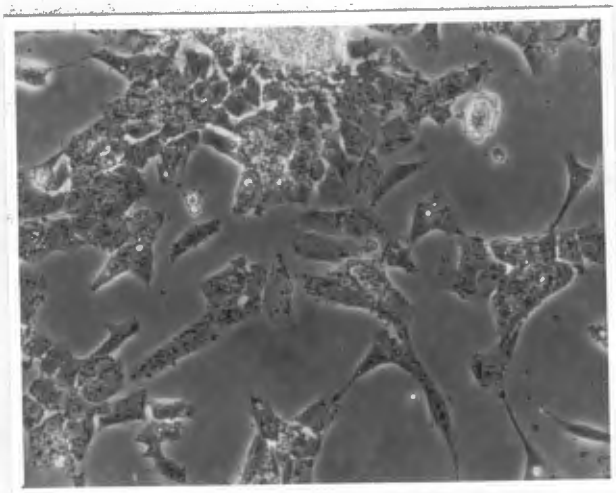
b. Experiments C/D.



(x 250)

Figure 36. DPH (5 μg/ml *) AND RETINOIC ACID (10^{-6}M)

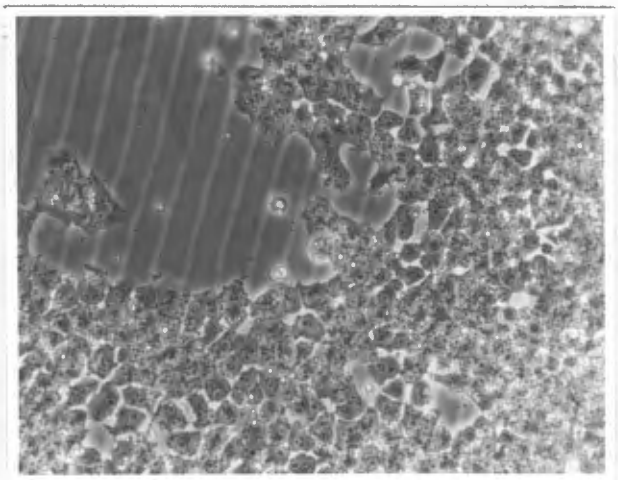
a. Experiment A.



(x 250)

Figure 37. DPH (500 μg/ml *) AND S-9 MIXTURE

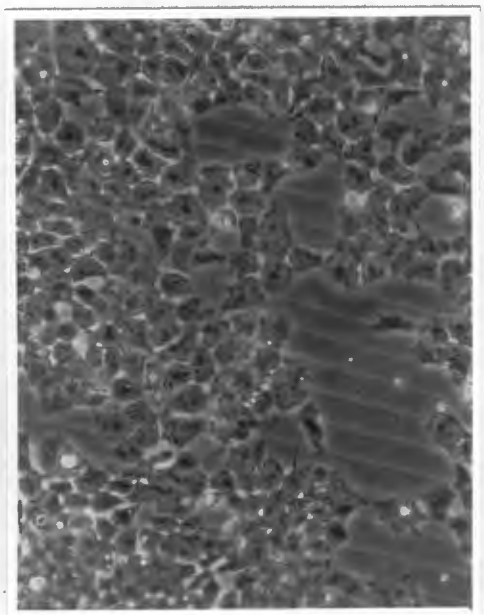
a. Experiment A.



(x 250)

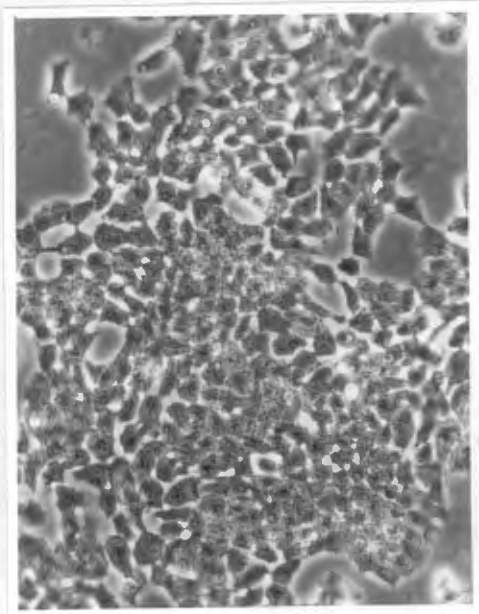
Figure 38. DPH (50 μg/ml *) AND S-9 MIXTURE

a. Experiments A/B.



(x 250)

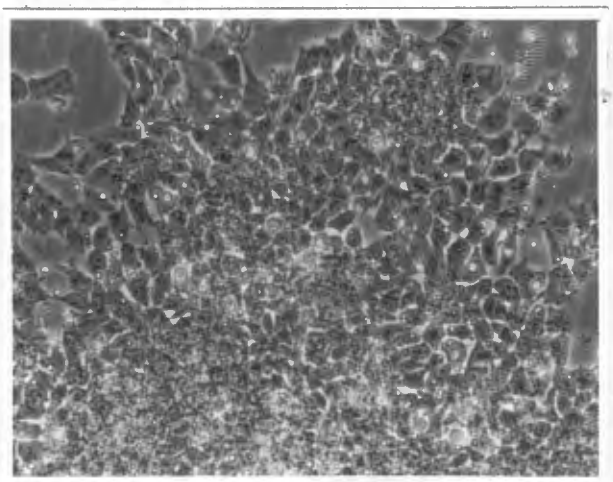
b. Experiments C/D.



(x 250)

Figure 39. DPH (5 μg/ml *) AND S-9 MIXTURE

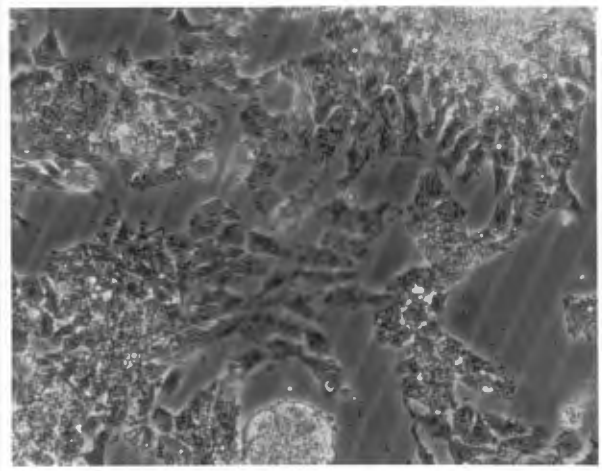
a. Experiment A.



(x 250)

Figure 40. DPH (500 μg/ml *), RETINOIC ACID (10^{-6}M) AND S-9 MIXTURE

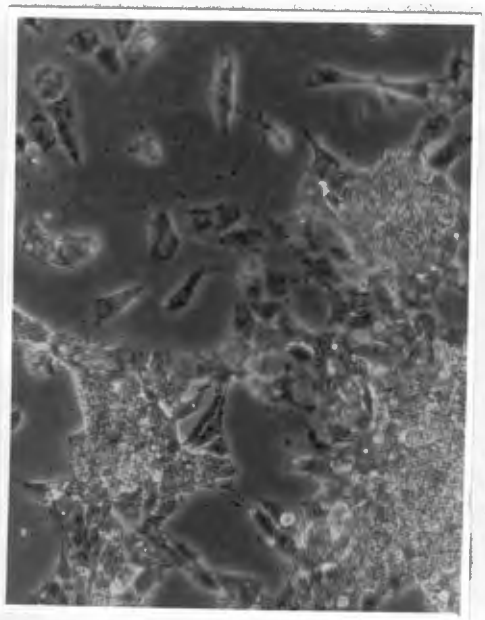
a. Experiment A.



(x 250)

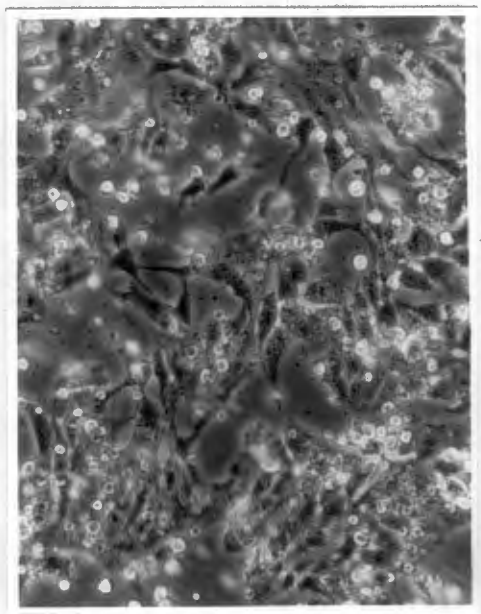
Figure 41. DPH (50 μg/ml *), RETINOIC ACID (10^{-6}M) AND S-9 MIXTURE

a. Experiments A/B.



(x 250)

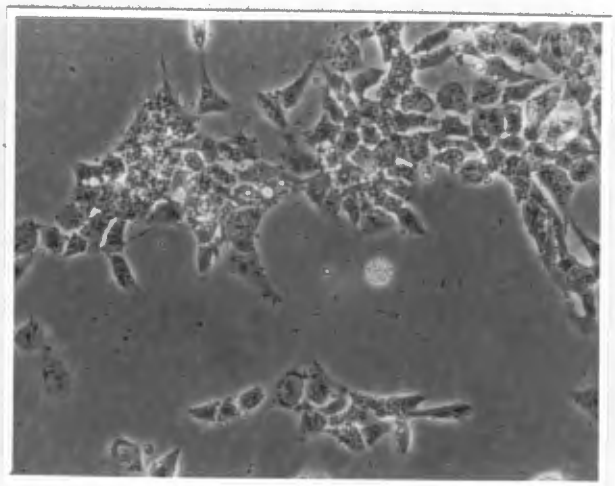
b. Experiments C/D.



(x 250)

Figure 42. DPH (5μg/ml *), RETINOIC ACID (10^{-6}M) AND S-9 MIXTURE

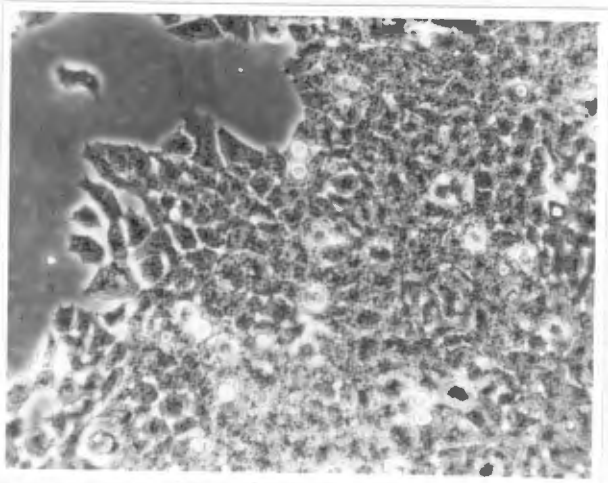
a. Experiment A.



(x 250)

Figure 43. DPH, m- HYDROXY METABOLITE (50μg/ml *)

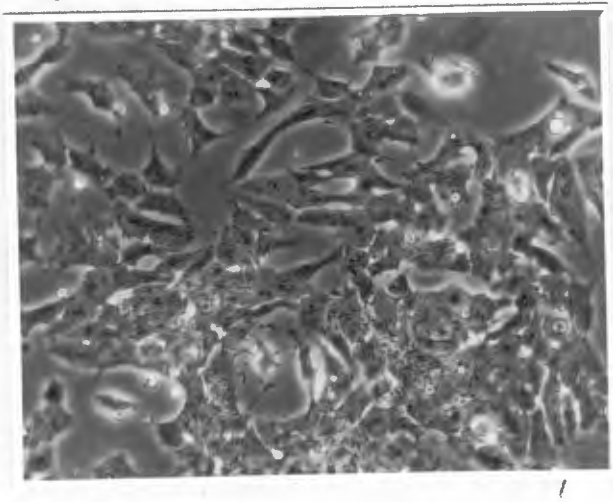
a. Experiment D.



(x 250)

Figure 44. DPH, m - HYDROXY METABOLITE (50 μg/ml *) AND RETINOIC ACID (10^{-6}M)

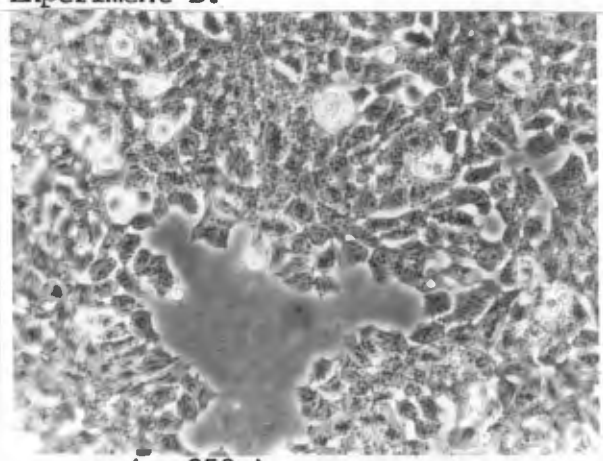
a. Experiment D.



(x 250)

Figure 45. DPH, p - HYDROXY METABOLITE (50 μg/ml *)

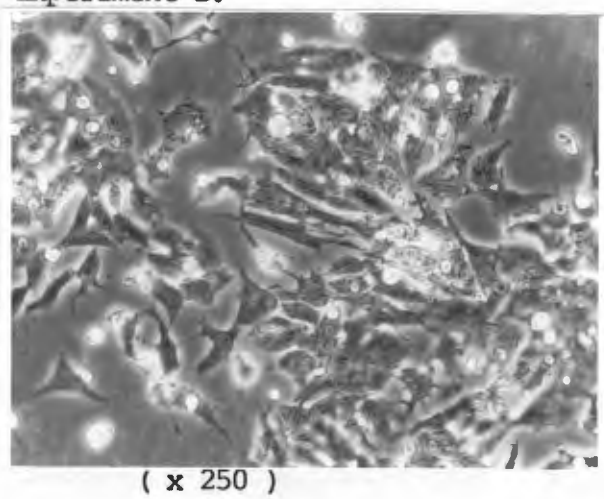
a. Experiment D.



(x 250)

Figure 46. DPH, p - HYDROXY METABOLITE (50 μg/ml *) AND RETINOIC ACID (10^{-6}M)

a. Experiment D.



(x 250)

7.5 PC13 MEAN CELL CYCLE TIME

The cells were in logarithmic growth when a straight line could be drawn through 3 plots (Figure 47.). The time was determined (horizontal scale) for the cell number to double (vertical scale) during log growth of the cells. This time approximated the mean population doubling time which in turn approximated the mean cell cycle time. For PC13 stem cells this was calculated to be between 18 and 20 hours.

Table 16. DETERMINATION OF THE GROWTH RATE OF PC13 EC CELLS IN CULTURE

Hours	Replicate CELL COUNTS (x 10^6): 1	2	3	4	5	6	7	8	AVERAGE
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
24	1.08	1.26	1.42	1.18	1.12	1.20	1.34	1.28	1.24
48	1.56	1.92	2.20	1.88	1.96	1.68	2.24	1.98	1.93
72	4.06	4.36	4.60	4.42	4.78	3.76	4.18	4.32	4.30
96	9.20	10.20	11.60	14.00	11.80	8.14	11.20	12.60	11.10

Figure 47. ESTIMATION OF THE MEAN CELL CYCLE TIME FROM POPULATION DOUBLING TIMES DURING LOGARITHMIC GROWTH OF PC13 EC CELLS IN CULTURE

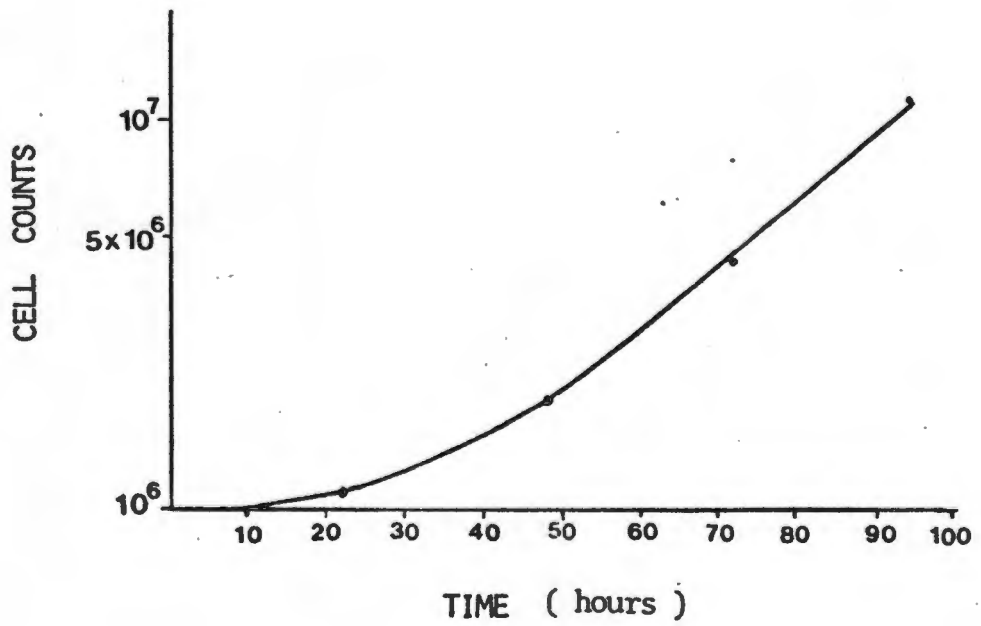


TABLE 17. THE NUMBER OF "HITS" FOR UNDIFFERENTIATED AND DIFFERENTIATING PC13 EC CELLS OUT OF A TOTAL OF 121 "HITS" BY THE 'POINT COUNTING METHOD'. (Refer to Section 6.21.2)

Treatment code	Treatment	EC**	DIFF***	EC**	DIFF***	EC**	DIFF***	EC**	DIFF***
1	Control	119	2	121	0	118	3	119	2
2	RA (10^{-6}M)	17	104	21	100	8	113	11	110
3	S-9 Mix	117	4	199	2	120	1	-	-
4	RA (10^{-6}M) and S-9 Mix	34	87	36	85	10	111	-	-
5	Solvent only	119	2	120	1	119	2	-	-
6	DPH (500 µg/ml*)	Cell death							
7	DPH (50 µg/ml*)	120	1	121	0	118	3	119	2
8	DPH (5 µg/ml*)	118	3	-	-	-	-	-	-
9	DPH (500 µg/ml*) and RA (10^{-6}M)	Cell death							
10	DPH (50 µg/ml*) and RA (10^{-6}M)	32	89	35	86	11	110	14	107
11	DPH (5 µg/ml*) and RA (10^{-6}M)	28	93	-	-	-	-	-	-
12	DPH (500 µg/ml*) and S-9 Mix	120	1	-	-	-	-	-	-
13	DPH (50 µg/ml*) and S-9 Mix	119	2	120	1	119	2	120	1
14	DPH (5 µg/ml*) and S-9 Mix	118	3	-	-	-	-	-	-
15	DPH (500 µg/ml*), RA (10^{-6}M) and S-9 Mix	41	80	-	-	-	-	-	-
16	DPH (50 µg/ml*), RA (10^{-6}M) and S-9 Mix	54	67	57	64	38	83	42	79
17	DPH (5 µg/ml*), RA (10^{-6}M) and S-9 Mix	60	61	-	-	-	-	-	-
18	DPH m-hydroxy metabolite (50 µg/ml)	-	-	-	-	-	-	119	2
19	DPH m-hydroxy metabolite (50 µg/ml) and RA (10^{-6}M)	-	-	-	-	-	-	12	109
20	DPH p-hydroxy metabolite (50 µg/ml)	-	-	-	-	-	-	118	3
21	DPH p-hydroxy metabolite (50 µg/ml) and RA (10^{-6}M)	-	-	-	-	-	-	10	111

NOTE: Results are of an average of data obtained on Day 12 (culture termination) using 8 culture flasks per treatment.
** = Undifferentiating PC13 EC cells.
*** = Differentiating PC13 EC cells.
* = approximate DPH dosages

TABLE 18. CELL DEATH, CELL COUNTS, AND % DIFFERENTIATION OF CELL CULTURES ON DAY 12 FOR EXPERIMENTS A, B, C AND D.

		Experiment A			Experiment B			Experiment C			Experiment D		
Treatment code	Treatment	Cell death	Cell count	% * differentiation	Cell death	Cell count	% * differentiation	Cell death	Protein (µg/ml) **	% * differentiation	Cell death	Protein (µg/ml) **	% * differentiation
1	Control	Low	2.1×10^8	<1	Low	1.7×10^8	0	Low	100.5	<1	Low	70.5	<1
2	Retinoic Acid (10^{-6}M)	Moderate	1.9×10^7	63.6	Moderate	1.0×10^8	57.6	Moderate	100.8	80.1	Moderate	86.0	74.1
3	S-9 Mix	Low	1.7×10^8	<1	Low	1.3×10^8	<1	Low	115.2	<1	———	———	———
4	Retinoic Acid (10^{-6}M) + S-9 Mix	High	5.2×10^7	42.2	High	3.9×10^7	40.3	High	57.3	76	———	———	———
5	Solvents	Low	2.0×10^8	<1	Low	1.4×10^8	<1	Low	81.0	<1			
6	DPH (High)	High	7.6×10^6	—	———	———	———	———	———	———	———	———	———
7	DPH (Intermediate)	Low	1.6×10^8	<1	Low	1.2×10^8	<1	Low	94.7	<1	Low	82.8	<1
8	DPH (Low)	Low	2.0×10^8	<1	———	———	———	———	———	———	———	———	———
9	DPH (High) + Ret. Acid (10^{-6}M)	High	1.4×10^5	—	———	———	———	———	———	———	———	———	———
10	DPH (Int.) + Ret. Acid (10^{-6}M)	Moderate	6.0×10^7	44.3	Moderate	4.8×10^7	41.2	Low	93.8	74.1	Low	68.8	68.6
11	DPH (Low) + Ret. Acid (10^{-6}M)	Moderate	7.7×10^7	48.7	———	———	———	———	———	———	———	———	———
12	DPH (High) + S-9	Low	1.0×10^8	<1	———	———	———	———	———	———	———	———	———
13	DPH (Int) + S-9	Low	1.5×10^8	<1	Low	1.1×10^8	<1	Low	47.0	<1	Low	88.8	<1
14	DPH (Low) + S-9	Low	1.9×10^8	<1	———	———	———	———	———	———	———	———	———
15	DPH (High)+Ret. Acid(10^{-6}M)+S-9	High	4.5×10^7	35.8	———	———	———	———	———	———	———	———	———
16	DPH (Int)+Ret. Acid(10^{-6}M)+S-9	High	4.0×10^7	26.2	High	3.1×10^7	24.3	High	38.0	38.4	High	27.7	35.0
17	DPH (Low)+Ret. Acid(10^{-6}M)+S-9	High	3.2×10^7	22.5	———	———	———	———	———	———	———	———	———
18	DPH, m-hydroxy metabolite	———	———	———	———	———	———	———	———	———	Low	83.2	<1
19	DPH, m-hydroxy metabolite+R.A. (10^{-6}M)	———	———	———	———	———	———	———	———	———	Moderate	73.2	72.2
20	DPH, p-hydroxy metabolite	———	———	———	———	———	———	———	———	———	Low	80.3	< 1
21	DPH, p-hydroxy metabolite+R.A. (10^{-6}M)	———	———	———	———	———	———	———	———	———	Moderate	89.0	76.0

NOTE:

* = % Differentiation calculated by using formulae in Section 6.21.2 and data in Table 15.

** = Refer to Section 6.29.2 for Lowry's method of Protein Determination.

DPH dosage in brackets - Refer to Table 13.
High = approximately 500 µg/ml
Intermediate = approximately 50 µg/ml
Low = approximately 5 µg/ml

TABLE 19 CELL DEATH, CELL DENSITY, AND % CELL DIFFERENTIATION DATA FOR EXPERIMENTS A, B, C AND D COMBINED.

(Summary of Table 17.)

Treatment code	Treatment	Figure	Cell death	Cell density	% Cell differentiation	Cell Morphology
1	Control	26	Low	High	<1%	Normal
2	Retinoic Acid (10^{-6}M)	27	Moderate	Moderate	69%	Differentiation
3	S-9 Mix	28	Low	High	<1%	Abnormal (Cell Retraction)
4	Retinoic Acid + S-9	29	High	Low	42.2%	Differentiation
5	Solvents	30	Low	High	<1%	Normal
6	DPH (500*)	31	Very High	Low	**	Abnormal (Cell Disintegration)
7	DPH (50*)	32	Low	High	<1%	Normal
8	DPH (5*)	33	Low	High	<1%	Normal
9	DPH (500*) + Retinoic Acid (10^{-6}M)	34	Very High	Low	**	Abnormal (Cell Disintegration)
10	DPH (50*) + Retinoic Acid (10^{-6}M)	35	Low/Mod.	Moderate	57%	Differentiation
11	DPH (5*) + Retinoic Acid (10^{-6}M)	36	Moderate	Moderate	49%	Differentiation
12	DPH (500*) + S-9	37	Low	High	<1%	Abnormal (Cell Retraction)
13	DPH (50*) + S-9	38	Low	High	<1%	Abnormal (Cell Retraction)
14	DPH (5*) + S-9	39	Low	High	<1%	Abnormal (Cell Retraction)
15	DPH (500*) + RA (10^{-6}M)+ S-9	40	High	Low	36%	Differentiation/Cell disintegration
16	DPH (50*) + RA (10^{-6}M) + S-9	41	High	Low	26%	Differentiation/Cell disintegration
17	DPH (5*) + RA (10^{-6}M)+ S-9	42	High	Low	23%	Differentiation
18	DPH, m-hydroxy metabolite	43	Low	High	<1%	Normal
19	DPH, m-hydroxy metabolite+RA (10^{-6}M)	44	Moderate	Moderate	72.2%	Differentiation
20	DPH, p-hydroxy metabolite	45	Low	High	<1%	Normal
21	DPH, p-hydroxy metabolite+RA (10^{-6}M)	46	Moderate	High	76%	Differentiation

Note:
**Attached cell debris prevented estimation

* Approximate DPH dosages in µg/ml (DPH metabolites at approximately 50 µg/ml)

7:8 DNA SYNTHESIS

The DNA synthesis rates (Table 20.) of each of the following treatments measured after a four hour period of incubation with ^{3}H - thymidine did not differ from controls; Retinoic acid only ($10^{-6}M$), S-9 Mixture only, Solvents only, and DPH only (approximately 50 μg/ml). This suggests that the above treatments did not have a toxic effect upon DNA synthesis. However, the following treatments were shown to differ significantly from controls; Retinoic acid ($10^{-6}M$) with S-9 Mixture, DPH (approximately 50 μg/ml) with Retinoic acid ($10^{-6}M$), DPH (approximately 50 μg/ml) with Retinoic acid ($10^{-6}M$) and S-9 Mixture, DPH, m- hydroxy metabolite (approximately 50 μg/ml) and finally DPH, m- hydroxy metabolite (approximately 50 μg/ml) with Retinoic acid ($10^{-6}M$). All but the last two treatments caused a significant decrease in the rate of DNA synthesis, whilst the latter two treatments actually caused a significant increase in the rate of DNA synthesis. DPH (approximately 50 μg/ml) with S-9 Mixture gave conflicting results: in Experiment C. there was a significant decrease whilst Experiment D. showed the treatment not to affect the DNA synthesis rate when compared with controls.

Table 20. WILCOXON STATISTICAL ANALYSIS OF DNA SYNTHESIS RATES : COMPARISON WITH CONTROLS

CODE (see Table 13.)	TREATMENT	EXPERIMENT B	EXPERIMENT C	EXPERIMENT D
2	Retinoic Acid (10^{-6}M)	+ (0.01%)	+ (0.01%)	+ (0.01%)
3	S-9 Mixture only		+ (0.01%)	
4	Retinoic Acid (10^{-6}M) and S-9 Mixture		- (0.01%)	
5	Solvents		+ (0.01%)	
7	DPH (Intermediate dose)*	+ (0.01%)	+ (0.01%)	+ (0.01%)
10	DPH (Intermediate dose)* and Retinoic Acid (10^{-6}M)	- (0.10%)	- (0.05%)	- (0.02%)
13	DPH (Intermediate dose)* and S9-Mixture		- (0.01%)	+ (0.01%)
16	DPH (Intermediate dose)*, Retinoic Acid (10^{-6}M) and S9-Mixture		- (0.01%)	- (0.01%)
18	m-hydroxy metabolite			- (0.02%)
19	m-hydroxy metabolite and Retinoic Acid (10^{-6}M)			- (0.10%)

Note: + = Null hypothesis accepted, - = Null hypothesis rejected * Approximate DPH dosages (metabolites added to approximately 50 µg/ml)

Treatments with DPH, p- hydroxy metabolite gave too small a sample size to be included in this analysis. (Drug in short supply)

7.91 CHROMOSOMAL ANALYSIS : CONVENTIONAL

Chromosomal analysis of PC13 EC cells revealed a chromosome count of between 38 to 42 chromosomes per metaphase (modal 41); the normal mouse chromosome complement is 40. This indicates that there has been a slight shift from normality during the development of these cells from a teratocarcinoma and subsequent animal passage and cell culturing.

The most frequent occurring chromosome number was 41 in virtually all treatments, except for; DPH only (5.3 µg/ml) in Experiment A, and DPH m-hydroxy metabolite (approximately 50 µg/ml) with Retinoic Acid (10^{-6}M) in Experiment D where the most frequently occurring chromosome number was respectively 42 or 41/42 occurring equally. However in these two instances, only between 60 and 100 metaphases were examined which was a relatively small sample size. Polyploidy was observed to a maximum of 4% of the total number of metaphases counted per treatment and the range for chromosome number recorded was 60 to 84 chromosomes per metaphase plate.

Four marker chromosomes were demonstrated in controls (Figures 49 to 52) and were as follows; a metacentric chromosome (M1), a very elongated acrocentric chromosome (M2), a large submetacentric chromosome (M3), and a small submetacentric chromosome (M4). Markers M1 and M2 have been G-banded and M1 appears to be an isochromosome (Figure 54).

Results were the same in all treatments and there was no increase in chromosome aberrations when monitored by the conventional chromosome analysis technique.

Table 21 .

TALLY SHEET : EXPERIMENT A.

Chromosome Number	38			39				40					41						42			Polyploid
Markers	M1	M2	M3	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1 M3	M3 M4	M1	M1 M2	M1 M3	
Treatments																						
1 A	0	0	0	0	1	2	0	0	0	2	2	3	6	0	16	3	1	2	1	8	1	2 (61 ; 80)
1 B	1	0	0	0	0	1	0	4	1	1	2	0	7	0	11	1	0	0	4	14	1	2 (60 ; 72)
Total	1	0	0	0	1	3	0	4	1	3	4	3	13	0	27	4	1	2	5	22	2	4
1 A	2	0	0	1	0	0	0	4	1	1	0	0	5	2	9	3	4	0	2	12	2	2 (82 ; 84)
1 B	1	0	0	1	0	0	0	5	2	1	0	0	5	0	12	4	1	0	2	13	2	1 (82)
Total	3	0	0	2	0	0	0	9	3	2	0	0	10	2	21	7	5	0	4	25	4	3
2 A	1	0	0	1	1	0	0	7	0	1	0	1	4	0	10	1	1	1	2	15	1	3 (67 ; 78 ; 82)
2 B	1	0	0	1	1	0	0	3	1	1	1	0	4	1	12	0	3	0	1	16	3	2 (78 ; 80)
Total	2	0	0	2	2	0	0	10	1	2	1	1	8	1	22	1	4	1	3	31	4	5
3 A	1	0	1	1	0	0	0	3	1	2	1	1	5	1	9	2	0	0	3	13	3	3 (76 ; 80 84)
3 B	0	1	0	2	0	0	0	4	0	2	0	0	8	1	10	2	1	0	4	10	4	1 (66)
Total	1	1	1	3	0	0	0	7	1	4	1	1	13	2	19	4	1	0	7	23	7	4
5 A	2	0	1	2	1	1	0	4	1	1	0	1	6	0	12	1	2	0	1	14	0	0
5 B	1	0	0	2	0	0	0	6	1	0	2	0	7	0	14	1	0	0	3	11	1	1 (84)
Total	3	0	1	4	1	1	0	10	2	1	2	1	13	0	26	2	2	0	4	25	1	1
7 A	2	0	0	3	0	0	0	5	1	1	1	0	7	0	10	2	1	0	3	11	1	2 (68 ; 79)
7 B	0	1	0	0	0	1	0	6	1	0	0	0	8	0	15	2	1	0	3	10	1	1 (64)
Total	2	1	0	3	0	1	0	11	2	1	1	0	15	0	25	4	2	0	6	21	2	3

Note: for Treatment code information, refer to Table 13.

Table 22. Continued/... TALLY SHEET : EXPERIMENT A.

Chromosome Number	38			39				40					41						42			Polyploid
Markers	M1	M2	M3	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1 M3	M3 M4	M1	M1 M2	M1 M3	
Treatment																						
8 A	1	1	0	1	1	0	0	5	0	0	0	1	10	0	9	1	0	0	2	14	3	2 (68 ; 70)
8 B	1	0	0	2	0	0	0	5	1	0	1	0	6	1	7	2	2	0	4	16	1	0
Total	2	1	0	3	1	0	0	10	1	0	1	1	16	1	16	3	2	0	6	30	4	2
10 A	3	1	1	1	0	0	2	3	0	2	0	1	5	1	12	2	0	0	4	12	2	1 (79)
10 B	1	0	0	2	0	0	0	5	1	0	1	0	7	1	4	2	2	0	4	14	2	2 (72 ; 84)
Total	4	1	1	3	0	0	2	8	1	2	1	1	12	2	16	4	2	0	8	26	4	3
11 A	1	0	0	2	1	0	0	6	0	0	1	0	11	0	8	2	0	0	3	11	1	1 (84)
11 B	1	0	0	3	0	0	0	8	1	0	1	0	6	0	12	1	0	0	3	13	1	2 (78 ; 80)
Total	2	0	0	5	1	0	0	14	1	0	2	0	17	0	20	3	0	0	6	24	2	3
12 A	2	0	0	3	0	0	2	3	1	0	0	0	4	0	12	2	1	0	5	11	1	3 (65 ; 74 ; 84)
12 B	0	1	0	4	0	0	1	4	0	1	1	1	5	1	10	2	0	0	4	14	1	0
Total	2	1	0	7	0	0	3	7	1	1	1	1	9	1	22	4	1	0	9	25	2	3
13 A	1	1	0	2	0	0	2	4	0	0	2	0	5	0	19	1	0	0	0	10	0	2 (68 ; 80)
13 B	2	0	0	2	0	0	0	5	0	1	1	1	6	0	12	1	2	0	2	14	3	1 (82)
Total	3	1	0	4	0	0	2	9	0	1	3	4	11	0	31	2	2	0	2	24	3	3
14 A	4	0	0	0	2	0	0	5	1	1	0	0	5	0	10	3	1	0	2	11	2	2 (76 ; 84)
14 B	1	1	0	3	0	0	2	3	1	0	3	0	6	0	12	0	2	0	5	10	1	0
Total	5	1	0	3	2	0	2	8	2	1	3	0	11	0	22	3	3	0	7	21	3	2

Table 23 .

TALLY SHEET : EXPERIMENT B.

Chromosome Number	38			39				40					41				42			Polyploid
Markers	M1	M2	M3	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1	M1 M2	M1 M3	
Treatments																				
1 A	1	0	0	2	0	0	0	0	0	1	3	0	4	1	4	0	2	11	0	1 (74)
1 B	1	0	0	1	0	0	1	2	1	0	2	0	3	0	5	0	0	13	1	0
Total	2	0	0	3	0	0	1	2	1	1	5	0	7	1	9	0	2	24	1	1
1 A	0	1	1	0	1	0	1	1	1	2	1	0	2	0	6	0	0	12	0	1 (80)
1 B	1	0	0	1	1	1	0	2	0	1	4	1	5	0	4	0	1	8	0	0
Total	1	1	1	1	2	1	1	3	1	3	5	1	7	0	10	0	1	20	0	1
2 A	0	0	0	0	0	0	0	1	0	1	4	0	6	0	7	1	1	8	0	1 (79)
2 B	0	1	1	2	1	0	1	1	0	2	2	0	3	0	5	0	0	10	0	1 (84)
Total	0	1	1	2	1	0	1	2	0	3	6	0	9	0	12	1	1	18	0	2
3 A	2	1	0	1	0	0	1	2	0	0	3	1	5	0	9	0	1	4	0	0
3 B	1	0	1	2	0	0	0	1	1	2	4	0	2	1	10	0	0	4	1	0
Total	3	1	1	3	0	0	1	3	1	2	7	1	7	1	19	0	1	8	1	0
5 A	1	0	0	1	0	1	2	1	2	0	4	0	5	0	3	0	1	8	0	1 (84)
5 B	0	1	0	1	0	1	0	1	0	1	2	0	3	0	5	0	1	14	0	0
Total	1	1	0	2	0	2	2	2	2	1	6	0	8	0	8	0	2	22	0	1
7 A	1	1	0	1	1	0	1	1	1	0	2	0	5	0	3	0	1	10	1	1 (82)
7 B	0	0	0	1	0	0	0	1	0	1	3	1	9	0	6	1	0	7	0	0
Total	1	1	0	2	1	0	1	2	1	1	5	1	14	0	9	1	1	17	1	1
10 A	1	0	0	1	1	1	0	2	1	1	1	1	5	0	2	1	0	11	0	1 (66)
10 B	1	1	0	1	1	0	1	1	0	0	3	0	4	0	5	1	0	10	0	1 (82)
Total	2	1	0	2	2	1	1	3	1	1	4	1	9	0	7	2	0	21	0	2
13 A	0	0	0	2	0	1	0	2	2	0	3	0	2	0	5	0	0	13	0	0
13 B	2	1	0	2	0	0	0	0	0	0	1	2	4	0	4	0	2	9	1	1 (68)
Total	2	1	0	4	0	1	0	2	2	0	4	2	6	0	9	0	2	22	1	1

Table 23 .

TALLY SHEET : EXPERIMENT C.

Chromosome Number	38				39				40					41					42			Polyploid
Markers	M1	M2	M3	M1 M2	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1 M3	M1	M1 M2	M1 M3	
Treatments																						
1 A	0	1	0	0	1	0	0	1	2	0	1	2	0	5	1	6	2	0	3	4	0	1 (67)
1 B	1	0	1	0	0	0	0	2	1	0	2	0	1	6	1	3	1	0	4	7	0	0
Total	1	1	1	0	1	0	0	3	3	0	3	2	1	11	2	9	3	0	7	11	0	1
1 A	2	0	0	1	0	1	0	2	0	0	0	3	0	4	0	4	1	1	1	9	0	1 (83)
1 B	0	0	1	0	1	1	0	0	1	2	0	1	0	8	1	2	1	0	4	5	1	1 (84)
Total	2	0	1	1	1	2	0	2	1	2	0	4	0	12	1	6	2	1	5	14	1	2
2 A	0	0	0	2	0	0	1	1	1	0	2	1	1	6	0	7	1	0	0	6	0	1 (82)
2 B	0	1	0	0	0	1	1	1	3	1	1	2	0	4	0	3	1	1	4	6	0	0
Total	0	1	0	2	0	1	2	2	4	1	3	3	1	10	0	10	2	1	4	12	0	1
3 A	1	0	0	0	2	1	0	1	1	2	1	0	0	6	0	2	0	0	5	8	0	0
3 B	0	1	0	2	0	0	0	2	0	1	1	0	0	5	1	5	1	0	3	8	0	0
Total	1	1	0	2	2	1	0	3	1	3	2	0	0	11	1	7	1	0	8	16	0	0
5 A	3	1	0	0	1	1	0	1	2	0	0	3	1	3	0	4	0	0	4	3	2	1 (72)
5 B	0	0	2	0	0	1	0	1	1	0	1	1	0	6	1	5	2	0	2	7	0	0
Total	3	1	2	0	1	2	0	2	3	0	1	4	1	9	1	9	2	0	6	10	2	1
7 A	0	2	1	0	0	1	2	0	1	0	0	0	0	4	0	5	0	0	3	10	0	1 (84)
7 B	0	0	0	1	1	0	1	1	0	0	0	2	1	4	0	3	2	1	5	7	1	0
Total	0	2	1	1	1	1	3	1	1	0	0	2	1	8	0	8	2	1	8	17	1	1
10 A	1	0	0	2	1	1	0	2	1	1	0	0	1	9	0	2	0	0	3	6	0	0
10 B	1	1	1	0	0	2	0	1	1	0	0	2	1	6	0	3	0	0	4	5	1	1 (80)
Total	2	1	1	2	1	3	0	3	2	1	0	2	2	15	0	5	0	0	7	11	1	1
13 A	1	0	2	0	0	0	1	1	1	1	3	0	1	6	0	2	3	0	0	6	2	0
13 B	0	1	0	0	1	0	0	0	1	0	0	1	1	4	0	7	0	1	3	8	0	2 (78 ; 84)
Total	1	1	2	0	1	0	1	1	2	1	3	1	2	10	0	9	3	1	3	14	2	2

Table 24.

TALLY SHEET : EXPERIMENT D.

Chromosome Number	38			39				40					41					42			
Markers	M1	M3	M1 M2	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1 M3	M1	M1 M2	M1 M3	Polyploid
Treatments																					
1 A	2	0	2	5	0	1	0	4	0	3	1	1	4	0	3	0	1	0	2	0	1 (80)
1 B	1	0	0	3	0	1	0	4	0	1	2	0	3	0	7	1	1	1	3	0	2 (72 ; 82)
Total	3	0	2	8	0	2	0	8	0	4	3	1	7	0	10	1	2	1	5	0	3
1 A	0	0	3	2	0	0	0	2	0	1	0	0	2	0	10	2	0	0	7	0	1 (68)
1 B	2	1	1	2	0	1	1	1	0	2	1	1	3	0	7	0	0	2	4	0	1 (83)
Total	2	1	4	4	0	1	1	3	0	3	1	1	5	0	17	2	0	2	11	0	2
2 A	0	1	2	1	0	0	1	1	1	1	0	0	5	0	8	1	2	0	5	0	1 (84)
2 B	0	1	0	1	1	0	3	0	0	1	0	0	2	0	5	0	0	4	9	1	2 (78 ; 80)
Total	0	2	2	2	1	0	4	1	1	2	0	0	7	0	13	1	2	4	14	1	3
7 A	0	0	1	2	0	1	0	2	0	4	1	2	1	0	6	0	0	0	7	0	1 (80)
7 B	1	0	2	0	0	1	2	0	0	0	1	0	3	0	14	0	0	2	4	0	0
Total	1	0	3	2	0	2	2	2	0	4	2	2	4	0	20	0	0	2	11	0	1
10 A	0	1	0	3	1	0	1	3	0	2	0	0	6	0	6	0	0	0	7	0	0
10 B	0	2	2	0	1	2	0	2	1	0	0	0	3	0	9	0	0	1	6	0	1 (84)
Total	0	3	2	3	2	2	1	5	1	2	0	0	9	0	15	0	0	1	13	0	1

Table 24 . Continued/... TALLY SHEET : EXPERIMENT D.

Chromosome Number	38			39				40					41					42			Polyploid
Markers	M1	M3	M1 M2	M1	M2	M3	M1 M4	M1	M2	M3	M1 M2	M1 M3	M1	M2	M3	M1 M2	M1 M3	M1	M1 M2	M1 M3	
Treatments																					
13 A	1	2	0	1	2	0	0	3	2	0	0	1	0	0	4	1	2	0	10	0	1 (84)
13 B	3	0	0	1	0	0	0	1	0	0	4	1	3	0	6	3	0	0	6	1	1 (82)
Total	4	2	0	2	2	0	0	4	2	0	4	2	3	0	10	4	2	0	16	1	2
18 A	0	2	0	1	0	0	1	1	2	0	0	0	6	0	8	0	0	2	6	0	1 (82)
18 B	0	0	2	2	1	0	3	0	0	0	1	0	2	1	7	0	0	1	9	0	1 (74)
Total	0	2	2	3	1	0	4	1	2	0	1	0	8	1	15	0	0	3	15	0	2
19 A	2	0	2	0	0	0	1	2	0	0	0	0	3	1	7	0	0	0	10	1	1 (74)
19 B	0	3	1	1	1	0	1	0	1	1	0	0	3	0	5	2	0	1	8	1	1 (84)
Total	2	3	3	1	1	0	2	2	1	1	0	0	6	1	12	2	0	1	18	2	2
20 A	0	0	2	1	1	0	1	1	2	2	0	1	0	1	8	0	2	0	7	1	0
20 B	0	0	0	0	1	2	0	1	0	4	0	1	3	2	3	0	2	0	9	1	1 (80)
Total	0	0	2	1	2	2	1	2	2	6	0	2	3	3	11	0	2	0	16	2	1
21 A	1	2	2	3	0	0	1	1	0	0	0	0	2	0	7	3	1	2	5	0	0
21 B	1	0	0	1	2	0	0	1	0	0	2	0	6	1	6	1	1	0	7	0	1 (66)
Total	2	2	2	4	2	0	1	2	0	0	2	0	8	1	13	4	2	2	12	0	1

Table 25 . SUMMARY TALLY SHEET FOR EXPERIMENTS A/B AND C/D

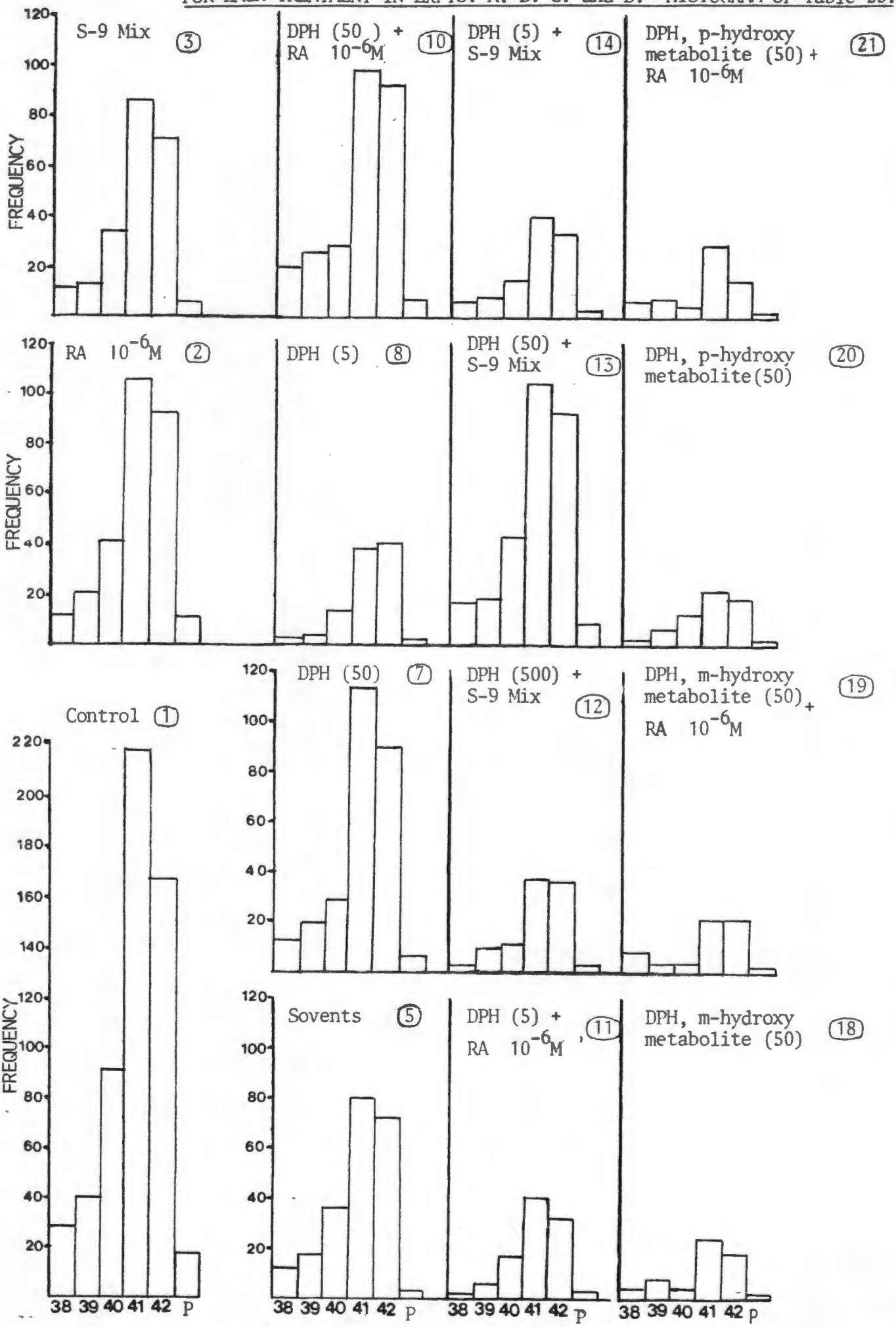
Chromosome Number	38					39					40						41							42				
Markers	M1	M2	M3	M1 M2	T	M1	M2	M3	M1 M4	T	M1	M2	M3	M1 M2	M1 M3	T	M1	M2	M3	M1 M2	M1 M3	M3 M4	T	M1	M1 M2	M1 M3	T	P
Treatments																												
1 A/B	7	1	1	0	9	6	3	4	2	15	18	6	9	14	4	51	37	3	67	11	6	2	126	12	91	7	110	9
1 C/D	8	1	3	7	19	14	2	3	6	25	15	2	10	10	3	40	35	3	42	8	3	0	91	15	41	1	57	8
Total					28*					40*						91*							217*				167*	17*
2 A/B	2	1	1	0	4	4	3	0	1	8	12	1	5	7	0	25	17	1	34	2	4	1	59	4	49	4	57	7
2 C/D	0	1	2	4	7	2	2	2	6	12	5	2	5	3	1	16	17	0	23	3	3	0	46	8	26	1	35	4
Total					11*					20*						41*							105*				92*	11*
3 A/B	4	2	2	0	8	6	0	0	1	7	10	2	6	8	2	28	20	3	38	4	1	0	66	8	31	8	47	4
3 C	1	1	0	2	4	2	1	0	3	6	1	3	2	0	0	6	11	1	7	1	0	0	20	8	16	0	24	0
Total					12*					13*						34*							86*				71*	4*
5 A/B	4	1	1	0	6	6	1	3	2	12	12	4	2	8	1	27	21	0	34	2	2	0	59	6	47	1	54	2
5 C	3	1	2	0	6	1	2	0	2	5	3	0	1	4	1	9	9	1	9	2	0	0	21	6	10	2	18	1
Total					12*					17*						36*							80*				72*	3*
7 A/B	3	2	0	0	5	5	1	1	1	8	13	3	2	6	1	25	29	0	34	5	2	0	70	7	38	3	48	4
7 C/D	1	2	1	4	8	3	1	5	3	12	3	0	4	4	3	14	12	0	28	2	1	0	43	13	27	1	41	2
Total					13*					20*						39*							113*				89*	6*
8 A	2	1	0	0	3*	3	1	0	0	4*	10	1	0	1	1	13*	16	1	16	3	2	0	38*	6	30	4	40*	2*

Table 25 . Continued/... SUMMARY TALLY SHEET

Chromosome Number	38					39					40						41							42				
Markers	M1	M2	M3	M1 M2	T	M1	M2	M3	M1 M4	T	M1	M2	M3	M1 M2	M1 M3	T	M1	M2	M3	M1 M2	M1 M3	M3 M4	T	M1	M1 M2	M1 M3	T	P
Treatments																												
10 A/B	6	2	1	0	9	3	3	1	3	10	9	1	4	6	3	23	18	1	27	5	3	0	54	8	47	4	59	5
10 C/D	2	1	4	4	11	4	5	2	4	15	7	2	2	2	2	5	24	0	20	0	0	0	44	7	25	1	33	2
					20*					25*						28*							98*				92*	7*
11 A	2	0	0	0	2*	5	1	0	0	6*	14	1	0	2	0	17*	17	0	20	3	0	0	40*	6	24	2	32*	3*
12 A	2	1	0	0	3*	7	0	0	3	10*	7	1	1	1	1	11*	9	1	22	4	1	0	37*	9	25	2	36*	3*
13 A/B	5	2	0	0	7	8	0	1	2	11	11	2	0	6	3	22	17	0	30	2	2	0	61	4	46	5	55	4
13 C/D	5	1	4	0	10	3	2	1	1	7	6	3	3	5	4	21	13	0	19	7	3	0	42	3	30	3	36	4
					17*					18*						43*							103*				91*	8*
14 A	5	1	0	0	6*	3	2	0	2	7*	8	2	1	3	0	14*	11	0	22	3	3	0	39*	7	22	3	32*	2*
18 D	0	0	2	2	4*	3	1	0	4	8*	1	2	0	1	0	4*	8	1	15	0	0	0	24*	3	15	0	18*	2*
19 D	2	3	3	0	8*	1	1	0	2	4*	2	1	1	0	0	4*	6	1	12	2	0	0	21*	1	18	2	21*	2*
20 D	0	0	2	0	2*	1	2	2	1	6*	2	2	6	0	2	12*	3	3	11	0	4	0	21*	0	16	2	18*	1*
21 D	2	2	2	0	6*	4	2	0	1	7*	2	0	0	2	0	4*	8	1	13	4	2	0	28*	2	12	0	14*	1*

Note: * = Cumulative Total for a particular treatment for Experiments A. B. C. and D.

Figure 48. COMBINED FREQUENCY TOTALS vs. CHROMOSOME COUNT (per metaphase) FOR EACH TREATMENT IN EXPTS. A. B. C. and D. HISTOGRAM OF Table 25.



NOTE: ( ) = approximate drug dosage in µg/ml, ○ = treatment code (Refer to Table 13)
p = polyploid.
RA = Retinoic Acid DPH = Diphenylhydantoin

TABLE 26. SUMMARY OF THE RESULTS OF CONVENTIONAL CHROMOSOME ANALYSIS FOR EACH TREATMENT IN EXPERIMENTS A,B,C AND D COMBINED.

Treatment code	Treatment	Total number of metaphases counted	Range of chromosome count per metaphase (excluding polyploids)	Most frequently occuring chromosome number over this range	Number of polyploids and range.
1	Control	560	38 - 42	41	17 (60 - 84)
2	RA (10^{-6}M)	280	38 - 42	41	11 (67 - 84)
3	S-9 Mix	220	38 - 42	41	4 (66 - 84)
4	RA (10^{-6}M) and S-9 Mix	-	-	-	-
5	Solvents only	220	38 - 42	41	3 (72 - 84)
6	DPH (500 µg/ml*)	-	-	-	-
7	DPH (50 µg/ml*)	280	38 - 42	41	6 (64 - 84)
8	DPH (5 µg/ml*)	100	38 - 42	42	2 (68 - 70)
9	DPH (500 µg/ml*) + RA (10^{-6}M)	-	-	-	-
10	DPH (50 µg/ml*) + RA(10^{-6}M)	270	38 - 42	41	7 (66 - 84)
11	DPH (5 µg/ml*) + RA (10^{-6}M)	100	38 - 42	41	3 (78 - 84)
12	DPH (500 µg/ml*) + S-9 Mix	100	38 - 42	41	3 (65 - 84)
13	DPH (50 µg/ml*) + S-9 Mix	280	38 - 42	41	8 (68 - 84)
14	DPH (5 µg/ml*) + S-9 Mix	100	38 - 42	41	2 (76 - 84)
15	DPH (500 µg/ml*),RA(10^{-6}M)+S-9 Mix	-	-	-	-
16	DPH (50 µg/ml*),RA(10^{-6}M)+S-9 Mix	-	-	-	-
17	DPH (5 µg/ml*), RA(10^{-6}M)+S-9 Mix	-	-	-	-
18	DPH m-hydroxy metabolite (50 µg/ml)	60	38 - 42	41	2 (74 - 82)
19	DPH m-hydroxy metabolite (50 µg/ml) and RA (10^{-6}M)	60	38 - 42	41/42	2 (74 - 82)
20	DPH p-hydroxy metabolite (50 µg/ml)	60	38 - 42	41	1 (80)
21	DPH p-hydroxy metabolite (50 µg/ml and RA (10^{-6}M)	60	38 - 42	41	1 (66)

Note: * = approximate Drug dosages

Figure 49. MARKER CHROMOSOMES: M1 (METACENTRIC) AND M4 (SMALL SUBMETACENTRIC)

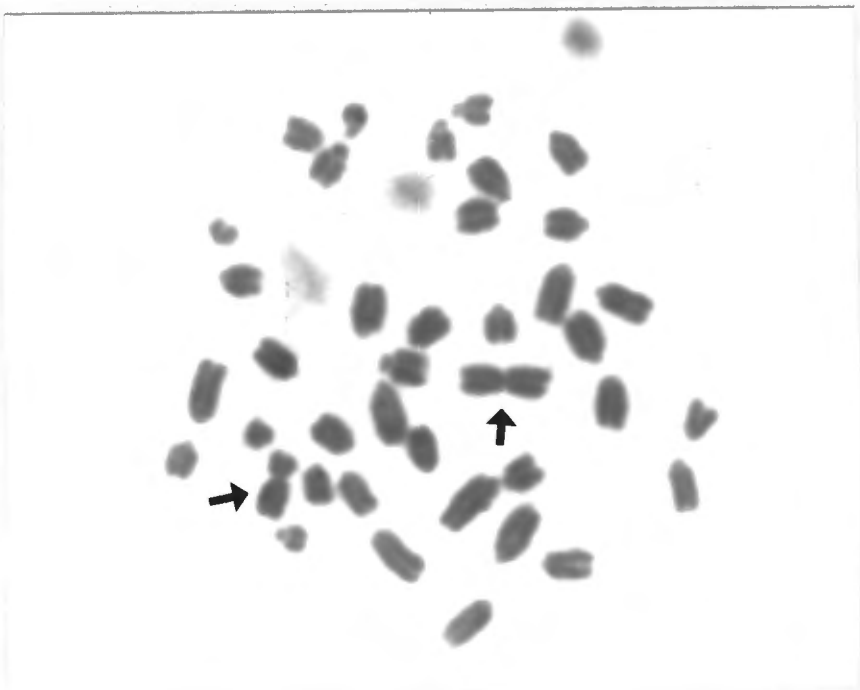


Figure 50. MARKER CHROMOSOMES: M1 (METACENTRIC) AND M2 (ELONGATED ACROCENTRIC)

Figure 51. MARKER CHROMOSOMES: M3 (LARGE SUBMETACENTRIC)

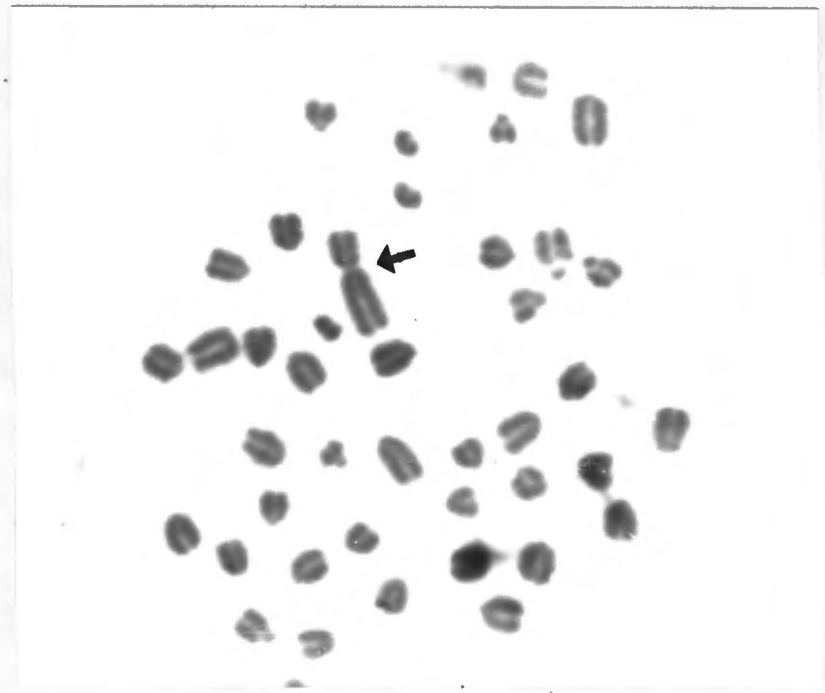


Figure 52. MARKER CHROMOSOMES: M3 (LARGE SUBMETACENTRIC) AND M4 (SMALL SUBMETACENTRIC)

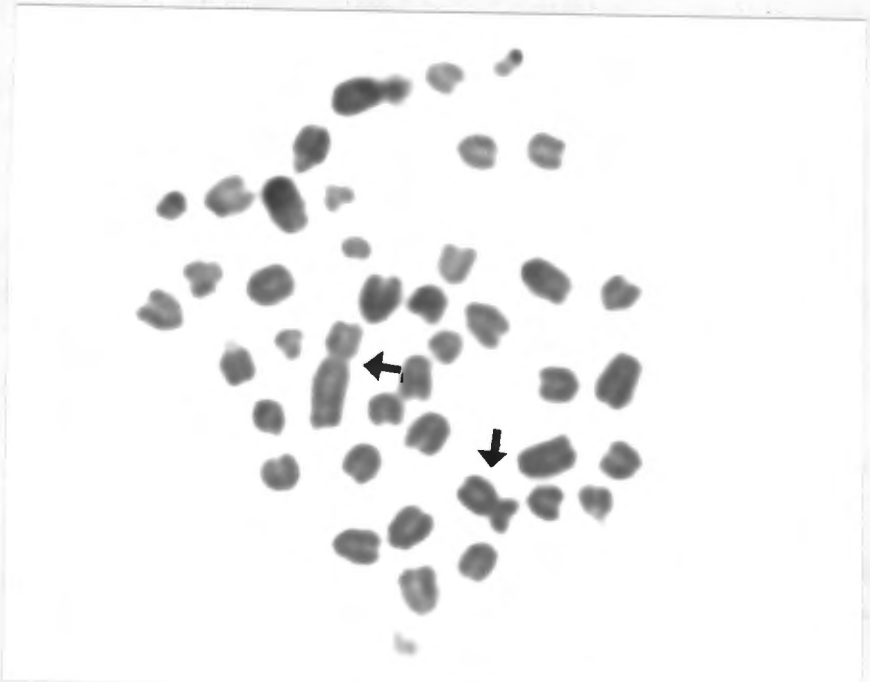


Figure 53. POLYPLOIDY (61 CHROMOSOMES): MARKER CHROMOSOMES - 2 x M1 (METACENTRICS) AND 2 x M4 (SMALL SUBMETACENTRICS)

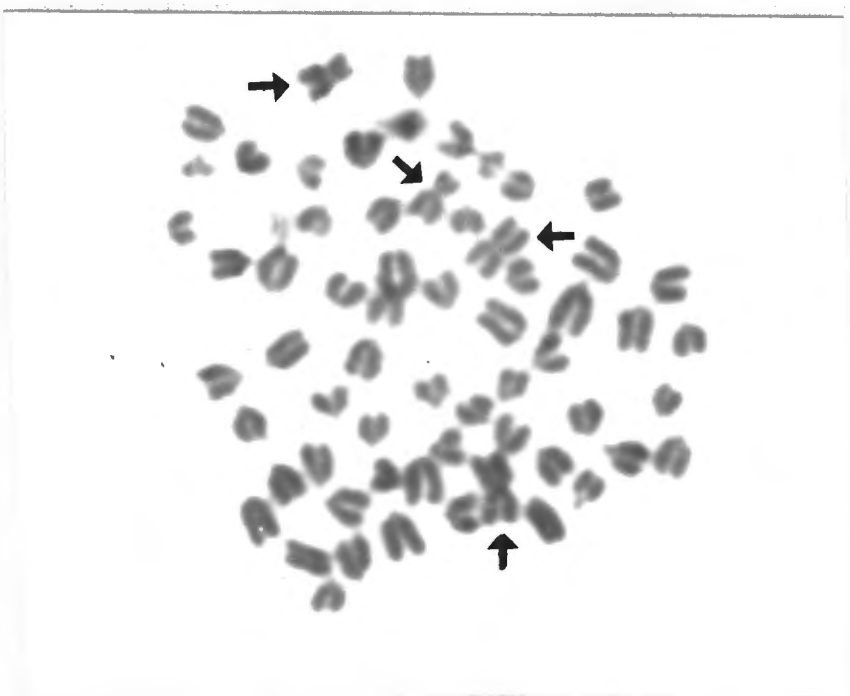
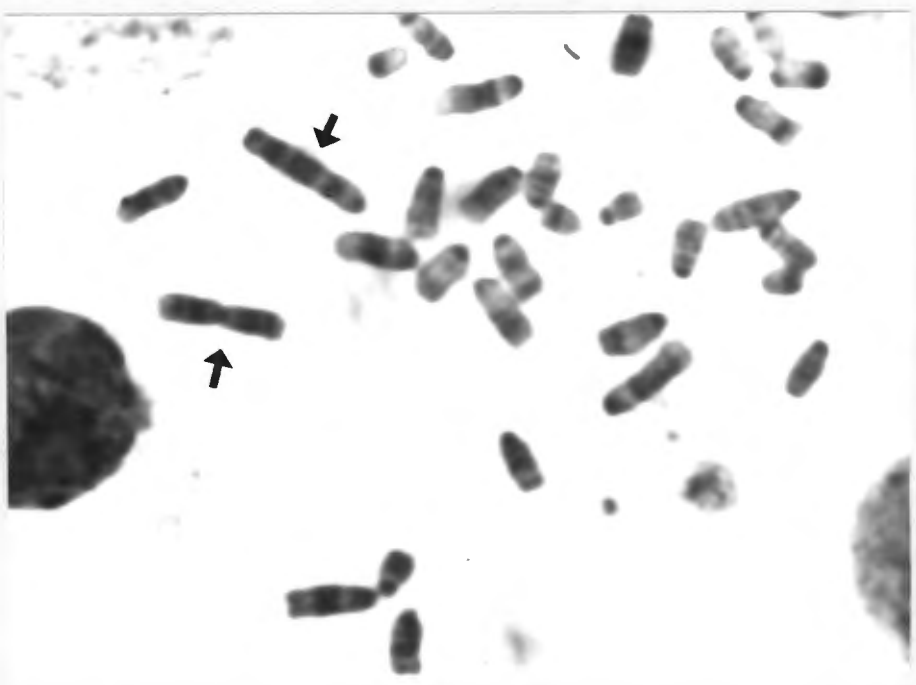


Figure 54. G - BANDING: MARKER CHROMOSOMES - M1 (METACENTRIC) and M2 (ELONGATED ACROCENTRIC)



The SCE frequency was also analysed and the most frequently occurring SCE number was 10 per metaphase (Figure 57) for all treatments including the controls, with two exceptions which were as follows; DPH (approximately 50 μg/ml) with Retinoic Acid (10^{-6}M), and DPH m-hydroxy metabolite (approximately 50 μg/ml) which had 9 and 11 respectively as the most frequent number of SCEs per metaphase. This seems to indicate that neither Retinoic Acid, DPH, S-9 Mixture, Solvents, nor the chemically synthesized metabolites in the amounts tested, are capable of increasing the SCE frequency rate above 12, the maximum for the controls.

BrdU has been shown to affect the SCE frequency (Bannigan and Langman 1979). Therefore a standard concentration of BrdU (10 μM) was used for controls and treatments which probably contributed to the baseline rate of SCEs which was found to range from 7 to 12 SCEs per metaphase. It was expected that an agent which could cause an increase in SCEs would be detected by causing an increase in the SCE frequency. A high number (18) of SCEs was infact observed when a cell culture was incubated in the presence of artificial light (Figure 56) and confirms the finding that physical factors such as light do influence the rate of SCE formation. All other cell cultures were incubated with BrdU in complete darkness and so this phenomenon was avoided but it did indicate that SCEs were inducible in the test system used.

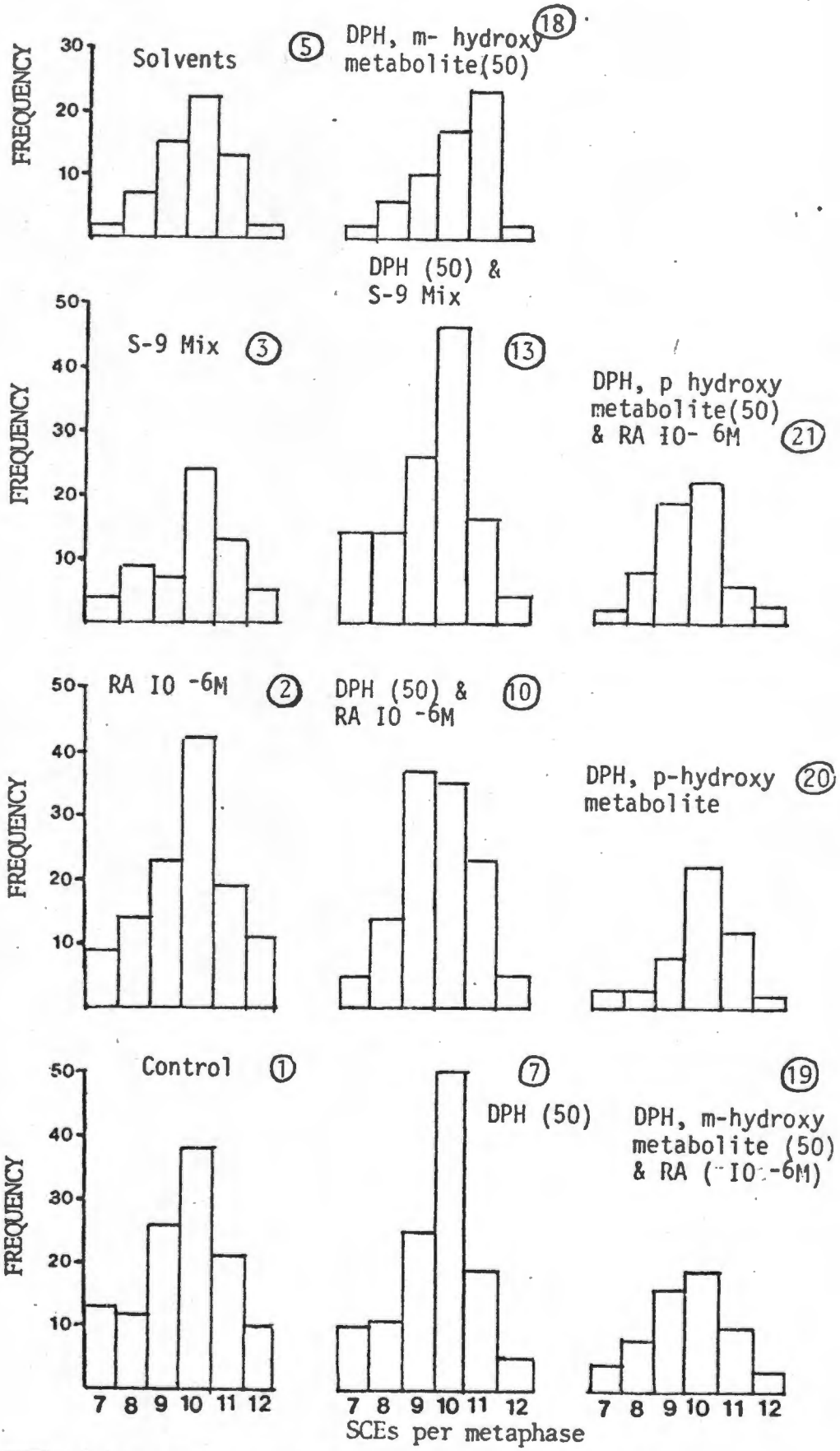
TABLE 27. SISTER CHROMATID EXCHANGE TALLY SHEET : EXPERIMENT C AND D.

Chromosome Number		38						39						40						41						42					
SCEs per Metaphase		7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12
Treatment code																															
EXPERIMENT C	1	1	2	0	1	0	0	2	1	0	3	0	1	3	1	4	4	0	0	4	0	6	9	4	5	1	1	2	5	0	0
	2	0	0	2	0	1	0	0	2	1	1	3	0	2	0	3	7	0	2	2	6	1	7	4	5	2	2	0	1	6	0
	3	0	1	0	2	0	0	2	0	4	1	1	0	1	2	0	5	0	0	1	3	1	14	12	3	0	3	2	2	0	2
	5	2	0	2	0	0	1	0	1	2	0	3	0	0	3	1	2	1	0	0	2	7	11	6	1	0	1	3	9	2	0
	7	0	1	1	0	1	0	1	0	0	2	1	0	2	1	0	5	2	1	1	1	4	15	3	2	3	1	2	7	3	0
	10	3	0	0	1	0	0	0	1	4	3	0	0	0	1	7	4	2	0	1	0	8	9	2	1	0	0	4	2	5	1
	13	1	3	0	2	1	0	2	0	1	4	0	0	2	2	0	4	1	0	3	2	4	12	6	2	0	2	3	3	0	0
EXPERIMENT D	1	0	1	2	0	2	0	1	3	0	1	1	0	0	3	2	3	2	1	1	0	3	10	8	2	0	0	7	2	4	1
	2	0	0	0	0	1	0	0	2	4	2	0	0	1	1	2	3	0	0	0	1	6	14	2	2	2	0	4	7	2	2
	7	0	0	1	1	0	0	1	1	0	1	2	0	1	1	4	6	1	0	1	4	12	10	3	2	0	1	1	3	3	0
	10	0	1	0	0	0	0	0	0	2	1	1	0	0	6	1	4	5	1	0	2	9	6	7	0	1	3	2	5	1	2
	13	0	1	0	2	1	0	0	0	1	1	1	0	3	1	4	3	0	0	3	2	8	7	4	1	0	1	5	8	2	1
	18	1	1	1	2	0	0	0	0	1	0	1	0	1	2	3	2	4	0	0	3	3	10	12	1	0	0	2	3	6	1
	19	2	0	0	2	2	0	2	3	1	2	0	1	0	1	5	3	1	0	0	2	8	6	7	2	0	2	2	6	0	0
	20	1	0	1	1	1	0	0	1	2	0	2	0	0	0	3	4	3	1	1	0	9	14	2	1	1	2	3	3	4	0
	21	0	0	1	1	0	0	0	1	2	1	0	0	0	3	2	6	4	0	2	1	5	8	0	1	0	3	9	6	2	2

Table 28 Combined frequency totals for the range of 38 to 42 chromosomes per metaphase and SCEs of between 7 and 12 in Experiments C and D.

Chromosome number (per metaphase)		38						39						40						41						42						Combined frequency totals per treatment					
SCE (per metaphase) frequency		7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12
TREATMENT CODE (Refer to Table 13)	1	1	3	2	1	2	0	3	4	0	4	1	1	3	4	6	7	2	1	5	0	9	19	12	7	1	1	9	7	4	1	13	12	26	38	21	10
	2	0	0	2	0	2	0	0	4	5	3	3	0	3	1	5	10	0	2	2	7	7	21	6	7	4	2	4	8	8	2	9	14	23	42	19	11
	3	0	1	0	2	0	0	2	0	4	1	1	0	1	2	0	5	0	0	1	3	1	14	12	3	0	3	2	2	0	2	4	9	7	24	13	5
	5	2	0	2	0	0	1	0	1	2	0	3	0	0	3	1	2	1	0	0	2	7	11	6	1	0	1	3	9	2	0	2	7	15	22	13	2
	7	0	1	2	1	1	0	2	1	0	3	3	0	3	2	4	11	3	1	2	5	16	25	6	4	3	2	3	10	6	0	10	11	25	50	19	5
	10	3	1	0	1	0	0	0	1	6	4	1	0	0	7	8	8	7	1	0	7	8	8	7	1	1	3	6	7	6	3	5	14	37	35	23	5
	13	1	4	0	4	2	0	2	0	2	5	1	0	5	3	4	7	1	0	6	4	12	19	10	3	0	3	8	11	2	1	14	14	26	46	16	4
	18	1	1	1	2	0	0	0	0	1	0	1	0	1	2	3	2	4	0	0	3	3	10	12	1	0	0	2	3	6	1	2	6	10	17	23	2
	19	2	0	0	2	2	0	2	3	1	2	0	1	0	1	5	3	1	0	0	2	8	6	7	2	0	2	2	6	0	0	4	8	16	19	10	3
	20	1	0	1	1	1	0	0	1	2	0	2	0	0	0	3	4	3	1	1	0	9	14	2	1	1	2	3	3	4	8	3	3	18	22	12	2
	21	0	0	1	1	0	0	0	1	2	1	0	0	0	3	2	6	4	0	2	1	5	8	0	1	0	3	9	6	2	2	2	8	19	22	6	3

Figure 55. COMBINED FREQUENCY TOTALS vs. SCE NUMBER (per metaphase) FOR EACH TREATMENT OF EXPERIMENTS C AND D (HISTOGRAM OF TABLE 28).



NOTE: ( ) = approximate drug dosage in µg/ml, ○ = treatment code (Refer to Table 13)
p = polyploid. RA = Retinoic Acid DPH = Diphenylhydantoin.

TABLE 29. SUMMARY OF SCE RATE FOR EXPERIMENTS C AND/OR D FOR EACH TREATMENT PERFORMED.

Treatment code	Treatment	Total number of metaphases counted	Range of chromosome count per metaphase (excluding polyploids)	Most frequently occuring chromosome number over this range	Most frequently occuring SCE rate
1	Control	120	38 - 42	41	10
2	RA (10^{-6}M)	120	38 - 42	41	10
3	S-9 Mix	60	38 - 42	41	10
4	RA (10^{-6}M) and S-9 Mix	-	-	-	-
5	Solvents only	60	38 - 42	41	10
6	DPH (500 µg/ml*)	-	-	-	-
7	DPH (50 µg/ml*)	120	38 - 42	41	10
8	DPH (5 µg/ml*)	-	-	-	-
9	DPH (500 µg/ml*) and RA (10^{-6}M)	-	-	-	-
10	DPH (50 µg/ml*) and RA (10^{-6}M)	120	38 - 42	41	9
11	DPH (5 µg/ml*) and RA (10^{-6}M)	-	-	-	-
12	DPH (500 µg/ml*) and S-9 Mix	-	-	-	-
13	DPH (50 µg/ml*) and S-9 Mix	120	38 - 42	41	10
14	DPH (5 µg/ml*) and S-9 Mix	-	-	-	-
15	DPH (500 µg/ml*), RA (10^{-6}M)+S-9 Mix	-	-	-	-
16	DPH (50 µg/ml*), RA (10^{-6}M)+S-9 Mix	-	-	-	-
17	DPH (5 µg/ml*), RA (10^{-6}M) + S-9 Mix	-	-	-	-
18	DPH m-hydroxy metabolite (50 µg/ml)	60	38 - 42	41	11
19	DPH m-hydroxy metabolite (50 µg/ml) and RA (10^{-6}M)	60	38 - 42	41	10
20	DPH p-hydroxy metabolite (50 µg/ml)	60	38 - 42	41	10
21	DPH p-hydroxy metabolite (50 µg/ml) and RA (10^{-6}M)	60	38 - 42	42	10

* = Approximate Drug dosage

Figure 56. SISTER CHROMATID EXCHANGE ANALYSIS: EXPOSURE TO ARTIFICIAL LIGHT DURING INCUBATION WITH BrdU - 18 SCEs

(Refer to page 53).

Figure 57. MODAL SISTER CHROMATID EXCHANGE RATE FOR ALL TREATMENTS IN EXPERIMENTS C AND D AFTER INCUBATION WITH BrdU IN COMPLETE DARKNESS - 10 SCEs

CHAPTER 8

DISCUSSION AND CONCLUSIONS

8.1 DISCUSSION

8.11 PC13 EC CELL DIFFERENTIATION IN CULTURE

A reproducible pattern of PC13 embryonal carcinoma cell differentiation was found to follow induction by low cell density plating (7×10^5 cells/culture flask) and exposure to Retinoic Acid (10^{-6}M). The sequence was as follows: the appearance of small granular Endoderm-like cells (20-30 μm) on days 2 to 4, Glial-like cells (30-60 μm) on days 4 to 5, Fibroblast-like cells (60-100 μm) from day 5, large Endoderm-like cells (100-200 μm) and multi-processed Neuronal-like cells (up to 900 μm) by day 18 to 21. Low cell density plating of 5×10^5 cells/culture flask gave similar results on day 21. The sequential and apparently limited pattern of differentiation was monitored by direct observation using phase contrast microscopy.

A comparison can be drawn between these differentiating cell types and the cell derivatives of the early endodermal germ layer which initially forms the epithelial lining of the primative embryonic gut, etcetera and the appearance of the large Endoderm-like cells similar to squamous epithelium would seem to confirm this (Langman 1981). The Fibroblast-like cells may represent a neuroectodermal derivative by analogy to early differentiation of the inner cell mass of the developing embryo. The Fibroblast-like cells may represent further glial differentiation, with glial tissue being a common component of well-differentiated murine teratocarcinomas in vivo (Kleinsmith and Pierce 1964) and Fibroblast-like cells have been described as resembling differentiating glial tissue of embryoid body differentiation in vitro (Van den Berg et al 1976). Focal neuronal

differentiation has been occasionally found associated with Fibroblast-like cells, which further suggests the possibility and it seems likely that these Fibroblast-like cells are analogous to cells of similar morphology in explants of embryonic brain tissue (Jones-Villeneuve 1982). However, it may be possible that these cells are a mixed population of morphologically similar cells representing more than one germ layer (Speers et al 1979). The reproducible sequence of differentiation after Retinoic Acid (10^{-6}M) treatment, with Glial-like and Fibroblast-like cells appearing early on followed by what appears to be large Neuronal-like cells is identical to that seen in explants of brain from 10-day old rat embryos (Jones-Villeneuve 1982).

Confirmation of the presence of a neuronal cell type requires data on neurotransmitter activity, neurite ultrastructure and sodium transport characteristics in response to certain neurotoxins (Pfeiffer et al 1981). Immunofluorescent staining of these neuronal type cells with antitubulin antiserum may reveal varicosities on some neuronal processes, a characteristic of some types of neurons. The Glial-like cells may be positively identified by staining with dye coupled with antibody to glial fibrillar protein (Jones-Villeneuve 1982). The identification of the biosynthetic product(s) of the giant cells in the future may help to clarify the relevance of this differentiation process to normal mouse embryogenesis in vivo (Lo and Guila 1980).

In summary, a definite identification of the cell types observed in this investigation was not made although the fact that undifferentiated EC cells appeared morphologically to be differentiating into more advanced cell types seemed to afford the criteria of 'embryonic-like development' required for teratogenicity testing.

The observations made on cell morphology after differentiation induction (Retinoic Acid 10^{-6}M) in experiments A, B, C and D confirmed the above sequence of events and the percentage of differentiating cells was on average 47% by day 7. The cells were then subjected to various treatments for the next five days which each affected the percentage of differentiation achieved, the degree of proliferation and the morphological appearance of cell cultures by day 12 (culture termination). The control cultures proliferated normally and did not change morphologically. In cultures where only retinoic acid (10^{-6}M) was present throughout, approximately 69% of the culture population was differentiating by day 12, with large Fibroblast-like cells (60-100 µm) predominating. Moderate cell death and density were recorded, indicating that Retinoic Acid (10^{-6}M) had a toxic effect, a result similar to that found by Linder et al (1981) and Ogiso et al (1982) using different cell systems. The other substances tested (viz. DPH and the S-9 mixture) did not cause the induction of cell differentiation when monitored by phase contrast microscopic analysis of cell morphology.

8.12 PC13 EC CELL EXPOSURE TO DPH (PARENT COMPOUND) AND ITS METABOLIC PRODUCTS (METABOLICALLY DERIVED USING S-9 MIXTURE AND CHEMICALLY SYNTHESIZED).

DPH at approximately 500 µg/ml had a very toxic effect upon undifferentiated and differentiating cells in culture, leading to a high rate of cell death and consequently to a lowered cell density (Table 18). The cells appeared granular and were surrounded by adherent debris, therefore it was not possible to observe their full morphology. DPH at 50 and 5 µg/ml caused low cell death and high cell density with normal cell morphology was therefore recorded for undifferentiated cell cultures. However, in differentiating cell cultures the degree of differentiation was reduced to

an average of 57% and 49% respectively instead of the expected 69% by day 12. There was also marked cell death, and consequently lowered cell density.

The addition of the S-9 mixture had no apparent effect on undifferentiated cell cultures except to cause the abnormal feature of cell retraction which is probably an indication of a toxic effect. Differentiating cells could not be assessed for this feature due to the inherent change in cell morphology with time, but there was a reduction in the percentage of cells differentiating to 42% instead of the expected 69% by day 12, implying some interference with the initiation of cell differentiation. It was found that the S-9 mixture added to undifferentiated cells with DPH at approximately 500 µg/ml appeared to provide protection against the expected drug toxicity, resulting in low cell death and consequently high cell density. DPH at 50 and 5 µg/ml with the S-9 mixture also led to a normal cell density and the only apparent effect on morphology was to cause cell retraction.

Differentiating cells (10^{-6}M Retinoic Acid induced) exposed to DPH at approximately 500 µg/ml in the presence of the S-9 mixture were however sensitive to DPH effects, although the effect was not as toxic as that which occurred with differentiating cells in the presence of DPH (approximately 500 µg/ml). This suggests that the conversion of DPH to its metabolites reduced toxicity similar to that with the same dosage of DPH and undifferentiated cells. It has similarly been found that DPH parent compound (50 µg/ml) once incubated with the S-9 mixture to induce the formation of metabolites has a much reduced toxic effect upon chick limb bud mesenchymal differentiation (Wilk et al 1980). It was concluded that the parent compound was the more toxic agent and that the S-9 mixture was possibly "protective" in converting DPH to its metabolites.

However in this investigation, the lower doses of DPH (50 and 5 µg/ml approximately) in the presence of the S-9 mixture had a more toxic effect (increased cell death, decreased cell density and reduced cell differentiation to 26 and 23% respectively) on differentiating cells than when no S-9 mixture was added.

The chemically synthesized metabolites, DPH m-hydroxy metabolite and DPH p-hydroxy metabolite had apparently no effect on undifferentiated cells but with differentiating cells they caused an increase in cell death and consequently lowered cell density. The morphology of the differentiating cells did not appear affected by the addition of these metabolites but they both caused a slight increase (to 72 and 76% respectively instead of 69%) in the degree of differentiation by day 12 when compared with the treatment of 10^{-6}M Retinoic Acid alone. It should be noted that an increase in protein synthesis and cell growth of cells obtained from enlarged rat gingiva resulted from prior treatment in vivo with up to 10 µg/ml of DPH but above this level, synthesis and growth were decreased (Hassell 1980). The significance of this result is not known and much thorough testing is required before firm conclusion can be made about the toxicity of these DPH metabolites.

8.13 THE EFFECT OF DPH ON DNA SYNTHESIS BY PC13 EC CELLS

DPH (approximately 50 µg/ml) was found not to cause a change from normal in DNA synthesis rates when administered as the parent compound to undifferentiated cells. The same result was found for Retinoic Acid (10^{-6}M) and the S-9 mixture when each was added alone. DPH (approximately 50 µg/ml) and S-9 mixture added together in Experiment C caused a significant decrease in the DNA synthesis rate but in Experiment D the results were not significantly different from those of the controls and therefore the initial

result suggesting DPH metabolic products caused some toxicity were not confirmed.

Retinoic Acid (10^{-6}M) and DPH (50 µg/ml) the parent compound caused a decrease in the DNA synthesis rate probably due to the toxic effect of Retinoic Acid mentioned previously or due to the formation of differentiating cells which may be more susceptible to DPH parent compound toxicity. These results were also observed when the S-9 mixture was included in the treatment, suggesting that both the parent compound or metabolites may be toxic to differentiating cells. Alternatively, it may be the Retinoic Acid (10^{-6}M) and S-9 mixture when added together which causes the toxicity in the presence of DPH since the S-9 mixture and Retinoic Acid (10^{-6}M) when added together without DPH caused a toxic effect which led to a decrease in the DNA synthesis rate.

The chemically synthesized DPH, m-hydroxy metabolite increased the DNA synthesis rate in undifferentiated and differentiating cells, implying that metabolites are toxic. The DPH, p-hydroxy metabolite was not available in an amount large enough to allow full analysis of its effect on the DNA synthesis rate.

8.14 DPH (PARENT COMPOUND AND METABOLIC PRODUCTS) EFFECTS ON CHROMOSOME ABERRATION AND SCE INDUCTION IN PC13 EC CELLS

The modal chromosome count was 41 per metaphase and the various treatments did not cause a significant change in this result and did not induce any chromosomal aberrations in addition to the four marker chromosomes already present in this cell line when analysed by conventional chromosomal methods. Therefore it is concluded that DPH doses of 50 µg/ml do not cause a mutagenic effect on PC13 EC cells in culture when monitored by chromosomal

analyses of aberration induction. This was the finding when DPH was administered as the parent compound, after being metabolically activated, and when two chemically synthesized metabolic products were added individually. Presumably, DPH acts at another level of DNA structure in order to exert its toxic effects and subsequent teratogenicity.

In addition, the SCE frequency was not increased above 12 SCEs per metaphase in all of the treatments suggesting that DPH, parent compound or metabolites does not cause a teratogenic effect by inducing SCEs.

8.2 CONCLUSIONS

1. The formation of possible neuronal components from PC13 EC cells was a protracted and more or less fortuitous process which has also been experienced by other investigators using a different clone of embryonal carcinoma cells (Kuff and Fewell 1980). A more suitable embryonal carcinoma cell line (such as PCC7) would provide a better test organism for the investigation of neuronal differentiation. At low cell densities, these cells preferentially differentiate into cholinergic neurons, making up at least 90% of the total cell population, the other cells being EC cells (Pfeiffer et al 1981). Such a cell line would be useful for the analysis of the process involved in the terminal differentiation of neurons, especially neurite outgrowth.

2. a) Results supporting the suggestion that the DPH parent compound is responsible for DPH toxicity are as follows:-

   i) DPH at approximately 500 µg/ml was toxic to undifferentiated cells in culture (increased cell death and decreased cell proliferation with abnormal cell morphology), however lower

doses (approximately 50 and 5 μg/ml) had no observable toxic effect. DPH at 50 μg/ml also had no effect on the rate of DNA synthesis.

ii) DPH appeared very toxic (increased cell death, decreased proliferation and decreased degree of differentiation) to differentiating cells at all three doses tested (approximately 500, 50 and 5 μg/ml). However, the 10^{-6}M Retinoic Acid used to induce differentiation may infact be responsible for some of the toxicity observed (especially at low DPH doses) even though the differentiating cells were thought to be more 'vulnerable', since Retinoic Acid alone causes increased cell death and decreased cell proliferation when compared with controls. DNA synthesis by differentiating cells had decreased with a DPH dose of approximately 50 μg/ml, whilst Retinoic Acid (10^{-6}M), was not found to cause a decrease in the DNA synthesis rate when tested alone.

iii) Incubation of DPH (parent compound at approximately 500, 50 and 5 μg/ml) with the S-9 mixture presumably led to the formation of metabolic products which had no apparent toxic effect (cell death and proliferation similar to controls) on undifferentiated cells, although 'cell retraction' was observed which can be attributed to the S-9 mixture since it occurs with S-9 mixture alone. This infers that at the higher DPH dose (approximately 500 μg/ml) especially, the S-9 has a protective effect in converting DPH to its metabolites and avoiding toxicity due to the high dose of the parent compound.

Conflicting results were found with regard to DNA synthesis rates and therefore will not be stated here.

iv) With differentiating cells (10^{-6}M Retinoic Acid induced), the DPH metabolic products formed in the presence of the S-9 mixture and DPH at approximately 500 µg/ml caused toxicity (increased cell death, decreased proliferation and decreased degree of differentiation (to 36%)). However, there was less toxicity when compared with the effect of DPH (approximately 500 µg/ml) on differentiating cells without the S-9 mixture, suggesting that the metabolic products are less toxic than the parent compound at high doses or alternatively, the S-9 mixture may not have caused adequate metabolic activation of the parent compound and the DPH parent compound may have been responsible for all the toxicity actually observed.

b) Results supporting the suggestion that DPH metabolites (resulting from metabolic activation of DPH or chemically synthesized) are responsible for DPH toxicity and not the parent compound are as follows:-

i) With differentiating cells (10^{-6}M Retinoic Acid induced), the DPH metabolic products formed in the presence of the S-9 mixture and DPH at 50 and 5 µg/ml (compare with 1(iv) above) were found to be more toxic (increased cell death, decreased cell proliferation and a decrease in the degree of cell differentiation to 26 and 23% respectively) than the parent compound at similar concentrations.

This implies that provided there was adequate metabolic activation of the DPH, the metabolic products are the cause of

DPH toxicity. In addition, a decreased rate of DNA synthesis by differentiating cells was observed with 50 µg/ml DPH in the presence of the S-9 mixture although this also occurred without the S-9 mixture, and also without the drug itself.

ii) The chemically synthesized metabolic products only (approximately 50 µg/ml) affected differentiating cells with regard to toxicity monitored as an increased in cell death, decreased cell proliferation and in increase in the degree of cell differentiation, however the DPH m-hydroxy metabolite was also found to cause increased level of DNA synthesis in both undifferentiated and differentiating cell cultures.

Overall, these results may therefore support the theory that DPH parent compound at therapeutic doses is able to cause toxicity to differentiating cells which can lead to teratogenic effects, although the experiment would need to be repeated using a larger sample size and perhaps a more controlled system of differentiation induction, metabolic activation of DPH and an improved sequence monitoring, before these conclusions can be made definite.

A positive control such as cyclophosphamide with the S-9 mixture has been recommended (Hsu.et al 1977) as an additional treatment which would ensure that metabolic activation was adequate and exclude inadequate activation of DPH where activated intermediates (arene oxides) or other metabolites were not formed leading to a negative result.. If DPH, the parent compound, is responsible for toxic effects it should be tested with a chemical not requiring metabolic activation,

for example, methylsulfonate which readily induces SCEs. Alternative positive controls may be required if the above two are not adequate to mimic the effect of DPH.

3. The negative response of undifferentiated and differentiating PC13 EC cells to the effects of DPH with regard to chromosome aberrations and SCE induction may be due to the possibility that EC cells are unable to respond due to various causes.

Fabricant and Hofnung (1979) who tested the effect of Mitomycin C (a known SCE inducer) on CBR2 EC cell cultures and due to a negative result concluded that the EC cell cultures may have been less permeable to the drug or defective in particular pathway of DNA repair. Further cytogenetic and biochemical analysis and the inclusion of definite positive and negative controls would probably resolve the problem of a possible false negative result in this investigation.

4. Some strains of mice have been shown to be more susceptible than others to the teratogenic effect of DPH (Mirkin 1971). Therefore as discussed, a more suitable murine teratocarcinoma cell line which may be more susceptible to DPH, and one in which, for example, definite neuronal differentiation predominates without chemical inducement should be examined. However, a human teratocarcinoma cell line with the above features would be the most appropriate.

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APPENDIX

The disappearance of DPH incubated with S-9 mixture (Refer to Table 11).
DPH initial amount = approximately 50 µg/ml.

◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇

PHENYTOIN

◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇

FOR LAST STD CURVE,

STD DEV = 1.76
DATE = 2.11
LOT #= 1202
PRESENT MODEL # = 1

SAMPLES OR
CALIBRATORS?

◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇◇

RUN SAMPLE # 3

Time = 0 (no S-9 mix)

Ao= 635
RATE = 307.1
(i) CONC (UG/ML) = 8.4 (x6)

RUN SAMPLE # 4
ID#?
Ao= 692
RATE = 308.9
(ii) CONC (UG/ML) = 8.9 (x6)

.....

RUN SAMPLE # 5

Time = 0 (with S-9 mix

Ao= 665
RATE = 299.1
(i) CONC (UG/ML) = 6.4 (x6)

RUN SAMPLE # 6

Time = 0 (with S-9 mix

Ao= 644
RATE = 300.1
(ii) CONC (UG/ML) = 6.7 (x6)

.....

RUN SAMPLE # 9

Time = 0.25 hrs

Ao= 719
RATE = 340.1
(i) CONC (UG/ML) = 24.4

.....

RUN SAMPLE # 11

Ao= 724
RATE = 338.7
(ii) CONC (UG/ML) = 23.3

.....

RUN SAMPLE # 13

Time = 0.5 hrs

Ao= 729
RATE = 326.1
(i) CONC (UG/ML) = 15.5

.....

RUN SAMPLE # 14

Ao= 731
RATE = 327.2
(ii) CONC (UG/ML) = 16.1

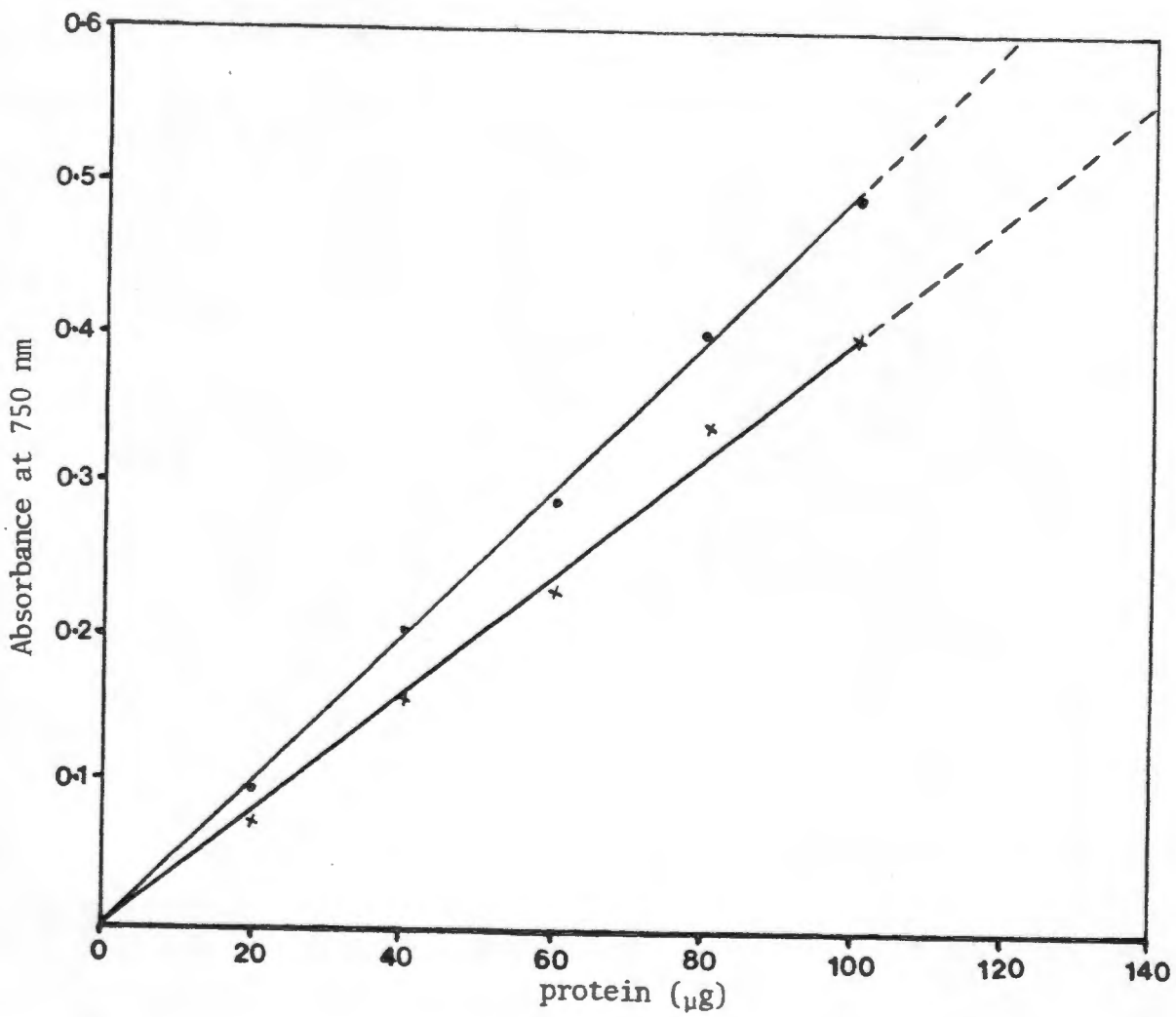
.....

```
     RUN SAMPLE #        4
                        Time = 1.0 hrs
     Ao=    628
     RATE =  315.0
 (i) CONC (UG/ML) =  10.8
     .....................
     RUN SAMPLE #        5

     Ao=    649
     RATE =  315.1
(ii) CONC (UG/ML) =  10.9

     RUN SAMPLE #       19
                        Time = 2.0 hrs
     Ao=    700
     RATE =  299.7
 (i) CONC (UG/ML) =   6.6
     .....................
     RUN SAMPLE #       20

     Ao=    734
     RATE =  298.2
(ii) CONC (UG/ML) =   6.2
     .....................
     RUN SAMPLE #       17
                        Time = 3.0 hrs
     Ao=    714
     RATE =  303.0
 (i) CONC (UG/ML) =   7.3
     .....................
     RUN SAMPLE #       18

     Ao=    743
     RATE =  310.2
(ii) CONC (UG/ML) =   9.3
     .....................
     RUN SAMPLE #        3
                        Time = 4.0 hrs
     Ao=    640
     RATE =  306.0
 (i) CONC (UG/ML) =   8.1
     .....................
     RUN SAMPLE #        4

     Ao=    674
     RATE =  301.7
(ii) CONC (UG/ML) =   7.0
     .....................
```

Standard curve of protein determinations for Experiments C and D:
Absorbances determined with increasing amounts of protein, assayed by the Lowry procedure.

Protein (µg/µl)	Experiment C (A)	(B)	AVERAGE	Experiment D (A)	(B)	AVERAGE
NIL	0.0004	0.0003	0.000	0.000(1)	0.000(4)	0.000
20	0.054	0.090	0.072	0.117	0.075	0.096
40	0.168	0.150	0.159	0.192	0.212	0.202
60	0.217	0.243	0.230	0.270	0.308	0.289
80	0.322	0.358	0.340	0.394	0.406	0.400
100	0.379	0.417	0.398	0.503	0.479	0.491

Standard curves for Experiments C (x) and D (•):



DNA Synthesis Rates: Liquid scintillation (dpm) estimations of ^{3}H-Thymidine incorporation.

```
PROG  1      USER  2

CNT CH  1    1 TIMES
CSS          1 TIMES
SCR =NO
AQC =YES
RCK =NO
CALC=        4
PST =    10.00 MIN
QCF =        0 NO
CH 1      1.00 2 SIGMA X
          50.0 LSR
          16.5 BKG
          1.00 2 SIGMA B
             0 LL
           428 UL


SINGLE LABEL DPM-VERS:08/01/76


UNKNOWN ID: 3H DPM
STANDARDIZATION ID: 3H #1563 BECKMAN
```

```
1. STANDARDS ID: 3H #1563 BECKMAN
2. UNK ID: 3H DPM
     UNKNOWN NORMALIZATION FACTOR:       1.00000000
3. DATA CHANNEL: 1
4. QUENCH MODE: H
    BKGRD CONSTANT QUENCH? Y
5. HALF LIFE(DAYS):      4492.5750
6. CALCULATE COEFF.? N
7. AVG BKGRD:
    CHAN #1:      19.8500
8. QUENCH COEFFS(A,B,C,D):
          60.550003,-    0.213265,-    0.001580,    0.00000677
9. QUENCH LIMITS(LOW,HIGH):     13.000000, 186.00000000
```

EXPERIMENT B

Treatments	D.P.M.
Control	893921.74
	548789.92
	199222.03
	346085.44
	254469.98
	326291.74
Retinoic Acid (10^{-6}M)	312403.53
	90598.07
	258037.33
	266980.05
	323840.36
	383681.55
DPH (50 µgm/ml)	256433.16
	254622.10
	282807.37
	165771.82
	190248.87
	919706.79
DPH (50 µgm/ml)	174097.23
	253208.77
Retinoic Acid (10^{-6}M)	267832.94
	190546.54
	251082.39
	198494.49

Note:

Only first 3 digits taken for calculations (Section 6.29.3) in DPM's (x thousand) and Results Table 20.

Experiment C. DNA Synthesis and Protein Determinations

Treatment:	(@750 nm) Average Absorbance	Protein (µgM)	D.P.M.	D.P.M. (Thousands per 100 µgM protein)
Control	0.403	102	162652.14	160
	0.448	111	159142.04	143
	0.280	71	118802.34	168
	0.537	134	196928.58	146
	0.352	88	143253.57	163
	0.382	96	173195.23	180
Retinoic Acid (10^{-6}M)	0.502	126	232920.73	185
	0.513	129	198101.66	154
	0.382	96	146407.18	152
	0.496	124	200319.95	161
	0.295	74	98875.20	134
	0.224	56	68386.73	121
S-9 mix	0.372	94	137067.51	146
	0.549	138	245326.35	178
	0.542	134	252960.92	188
	0.367	91	144994.62	159
	0.383	96	135755.87	142
	0.548	137	189268.17	137
Retinoic Acid (10^{-6}M) + S-9 mix	0.230	58	50019.25	86
	0.202	51	33726.09	67
	0.198	50	42039.28	82
	0.266	67	89848.01	134
	0.214	43	56028.25	130
	0.298	74	96478.31	129
Solvents	0.263	66	121131.00	183
	0.299	75	109988.13	145
	0.278	70	111894.47	160
	0.295	74	104237.05	141
	0.266	67	108430.23	161
	0.529	133	191341.58	144
DPH (interm) *	0.352	88	126425.00	143
	0.544	137	208352.95	152
	0.362	91	134222.49	147
	0.442	111	144962.53	131
	0.291	73	104975.27	144
	0.270	68	110026.41	162
DPH (interm) * and Ret. Acid (10^{-6}M)	0.548	138	192200.76	139
	0.538	135	220765.55	164
	0.409	103	149396.30	141
	0.494	125	153475.74	123
	0.171	43	21341.69	49
	0.076	19	6149.98	32

(contd. next page...)

DPH (interm)* and S-9 mix	0.228	57	43105.78	75
	0.216	54	12531.13	24
	0.204	51	17640.86	35
	0.160	40	14893.95	38
	0.169	41	30606.53	74
	0.158	37	25088.22	66
DPH (interm)* + S-9 mix and Retinoic Acid (10^{-6}M)	0.478	120	55221.22	45
	0.154	38	11217.92	28
	0.042	10	1653.42	20
	0.056	14	2015.51	14
	0.034	8	1906.91	25
	0.146	36	6626.83	19

* = Approximately 50 µg/ml

Experiment D. DNA Synthesis and Protein Determinations

Treatment:	(@750 nm) Average Absorbance	Protein (µgM)	D.P.M.	D.P.M. (Thousands per 100 µgM protein)
Control	0.284	57	44025.13	77
	0.310	62	39830.50	64
	0.298	60	34615.80	58
	0.369	74	63084.30	85
	0.357	72	52564.03	74
	0.480	97	92148.69	95
Retinoic Acid (10^{-6}M)	0.435	88	53755.18	61
	0.340	69	65065.52	94
	0.450	91	69787.07	77
	0.437	88	64065.97	73
	0.428	86	55693.76	65
	0.465	94	65547.96	70
DPH (50 µg/ml)*	0.400	81	78125.69	95
	0.321	64	42903.92	66
	0.484	98	72808.26	75
	0.535	108	72447.45	68
	0.350	70	42625.18	61
	0.362	73	61704.20	85
DPH (50 µg/ml)* Retinoic Acid (10^{-6}M)	0.328	66	34013.32	52
	0.303	61	39154.39	64
	0.357	72	28847.74	40
	0.328	66	27511.12	44
	0.248	50	24833.45	50
	0.485	98	55287.11	56
DPH (50 µg/ml)* S-9 Mix	0.446	90	63361.39	70
	0.437	88	65165.99	74
	0.390	79	41222.21	52
	0.406	82	61415.97	74
	0.484	98	51508.98	53
	0.475	96	81812.20	85
DPH (50 µg/ml)* and Retinoic Acid (10^{-6}M) with S-9 Mix	0.120	24	12642.28	50
	0.150	30	15490.33	53
	0.152	38	17874.70	47
	0.165	33	14164.37	42
	0.104	21	7872.70	38
	0.101	20	8276.75	40
m-hydroxy metabolite (50 µg/ml)*	0.496	100	189216.12	189
	0.350	91	87071.98	96
	0.238	58	51906.28	90
	0.436	88	92214.14	105
	0.470	96	127796.41	133
	0.328	66	58062.19	88

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m-hydroxy metabolite and Ret. Acid (10^{-6}M)	0.308	62	54843.98	89
	0.336	68	47863.98	71
	0.262	53	51814.15	98
	0.387	78	88226.35	113
	0.440	88	108376.09	123
	0.445	90	116421.62	129
p-hydroxy metabolite (50 µg/ml)*	0.426	86	85257.51	99
	0.366	72	57525.91	81
	0.410	83	74614.29	90
p-hydroxy metabolite and Ret. Acid (10^{-6}M)	0.418	84	98509.15	118
	0.475	96	118984.71	124
	0.430	87	80585.02	93

* = Approximate values

CHROMOSOME ABERRATION : SCORE SHEET

TREATMENT :

CELL NUMBER	CHROMOSOME COUNT	MARKERS				OTHER
		M1	M2	M3	M4	
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2						
3						
4						
5						
6						
7						
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8 FEB 1985

SISTER CHROMATID EXCHANGE : SCORE SHEET

TREATMENT :

CELL NUMBER	CHROMOSOME COUNT	SCEs PER METAPHASE
1		
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